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Original article

Apoptosis in myelodysplasia: Association with patient age, bone marrow cellularity and karyotypes

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Abstract

Background: Myelodysplastic syndrome (MDS) comprises a heterogeneous group of clonal hematopoietic stem cell diseases, characterized by dysplasias and apoptosis in bone marrow (BM) and cytopenias in peripheral blood. In this study, we analyzed apoptosis in MDS to verify associations with patient age, bone marrow cellularity and karyotypes and to investigate the role of apoptosis in MDS pathogenesis. Methods: Bone marrow cells were collected from 81 patients with primary MDS, of which 60 were adults and 21 children. BM cells were also collected from 10 healthy donors for bone marrow transplants, 5 adults and 5 children, as controls. The patients and controls came from public onco-hematology institutions in Rio de Janeiro. The percentage of apoptotic BM cells was assessed by flow cytometry using two combinations: annexin V-FITC/CD34PE/CD45PerCP and annexin V-FITC/CD14PE/CD45PerCP in BM cells. Cytogenetic analysis was performed by G-banding. Results: The comparison between adult and pediatric patients showed that these patients show a similar behavior with regard to apoptotic cells percentages in BM samples. Apoptosis occurs independently of BM cellularity, being more prominent in patients with hyper/normocellular BM. Patients with normal karyotypes, del(5q), del(17p) had higher apoptosis rates than patients with del(11q) and complex karyotypes. Cells committed to a differentiation program were associated with high rates of apoptosis, suggesting that apoptosis may be a consequence of inefficient hematopoiesis, such that the hematopoietic system may eliminate dysplastic cells at the beginning of the disease. Conclusions: Our results suggest that apoptosis is an important characteristic of BM cells from adult and pediatric MDS patients and may be a consequence of inefficient hematopoiesis. In addition, we suggest that apoptosis is not the main mechanism associated with hypocellular MDS, and it occurs preferentially in MDS cases of hyper/normocellular BM and is associated with a good prognosis.

Keywords: Myelodysplastic syndrome; Apoptosis; Patient age; Bone marrow cellularity; Karyotypes.

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Introduction

Apoptosis or programmed cell death is an essential physiological process that plays a critical role in development and tissue homeostasis. Apoptotic cells may be characterized by specific morphological and biochemical changes, including cell shrinkage, chromatin condensation and internucleosomal cleavage of genomic DNA.[1] Apoptosis is involved in a wide range of pathological conditions. Cancer is one of the scenarios where little apoptosis occurs. The apoptosis signaling pathway plays an important role in the treatment of cancer because it is a target of many treatment strategies.[2,3]

Myelodysplasia or myelodysplastic syndrome (MDS) encompasses a group of clonal stem cell disorders characterized by dysplasias and apoptosis in bone marrow (BM), which lead to cytopenias in peripheral blood and a risk of progression to acute myeloid leukemia (AML). Peripheral cytopenias are known hallmark of MDS and they are associated with ineffective hematopoiesis, a condition in which the

BM is unable to produce and deliver adequate numbers of mature cells to the peripheral blood. Cumulative evidence indicates that this apparent paradox is caused by premature intramedullary cell death via apoptosis.[4] Some studies consider apoptosis in MDS as a defect that causes cytopenias in the peripheral blood, and apoptosis to be deregulated.[4-8] However, other studies point to apoptosis as a mechanism whereby the hematopoietic system is able to abrogate defective and/or potentially harmful clones.[9,10] Apoptosis in MDS remains unclear.[11]

MDS is viewed as a disease of adults, particularly the elderly. Pediatric MDS is an uncommon disorder, comprising less than 5% of hematopoietic malignancies.[12,13] In children, MDS appears with distinct clinical and laboratory characteristics when compared with adults, which may reflect specific biological issues related to MDS during childhood.[14] However, studies do not show the frequency of apoptosis in pediatric patients or if this process is similar when compared with BM cells from adult MDS patients. Another important point, focusing on apoptosis, concerns BM cellularity in MDS patients. The BM in primary MDS patients is usually hypercellular or normocellular. Nevertheless, between 10% and 20% of patients can present hypocellular BM.[15-17] Many studies involving apoptosis in MDS have been conducted in patients with hypercellular BM. In hypocellular MDS, it is unclear if apoptosis has some influence in this low cellularity. Goal and colleagues[18] suggested that hypocellularity in MDS could be explained by excessive apoptosis, but they also suggested that there is another sub-group of patients who may have a stem-cell failure defect since they show no evidence of apoptosis.

In primary MDS, independently of cellularity in the BM, the cytogenetic pattern is characterized mainly by partial or total loss of chromosomes.[19] Few studies show an association between apoptosis and karyotypes. The presence of del(5q) and trisomy 8 have been associated with a reduction in the rate of apoptosis in MDS.[20,21] The aim of this study was to analyze apoptosis in primary MDS in order to verify if there are associations with patient age, bone marrow cellularity and karyotypes and to investigate the role of apoptosis in MDS pathogenesis.

Materials and methods

Patients and controls

Bone marrow cells were collected from 81 patients with primary MDS. These patients included 34 males and 47 females. There were 60 adult patients, with the mean age of 54 years, ranging from 21-86 years, and 21 pediatric patients, with the mean age of 10 years, ranging from 5 months to 18 years. The patients were diagnosed at the following public hematology/oncology centers in Rio de Janeiro, Brazil: Bone Marrow Transplant Center (in Portuguese, Centro de Transplante de Medula Óssea, CEMO-INCA), Hematology Service (in Portuguese, Instituto Nacional do Câncer, INCA), Arthur Siqueira Cavalcanti Hematology Institute (in Portuguese, HEMORIO) and Martagão Gesteira Pediatric and Puericulture Institute (IPPMG). The criteria for inclusion of the patients were the presence of dysplastic cells and the percentage of blasts according to the bone marrow analysis and immunophenotyping. The diagnosis was based on clinical history, morphological, cytochemical studies, immunophenotypic and cytogenetic analyses. None of the patients had been previously treated for a malignancy. The pediatric patients included individuals aged 18 years or less, while adult patients were those aged 19 years or more. The pediatric patients were classified according to Hasle and colleagues.[22] Seventeen pediatric patients were classified as having refractory cytopenia (RC) and four as having refractory anemia with excess of blasts (RAEB). The classification of adult patients was in accordance with the French-American British (FAB) Co-operative Group.[23] Forty-six adult patients were classified as having refractory anemia (RA), eleven patients as RAEB and three as RAEB in transformation (RAEB-t). The cellularity of the bone marrow was analyzed by means of biopsy. The age-related normal values of bone marrow cellularity (%) were: infant (1 month to 1 year): 80-90%; child: 60-80%; adult: 40-70%; and ≥70 years: 30-40%.[24-26] The bone marrow samples from pediatric and adult MDS patients were sent to the Cytogenetic and Immunology Laboratories of CEMO-INCA, at the time of diagnosis. The cytogenetic analyses were conducted at the Cytogenetic Laboratory (CEMO-INCA) and the immunophenotyping and apoptosis experiments were conducted at the Immunology Laboratory (CEMO-INCA). The interpretation and discussion of apoptosis were performed at IPPMG-UFRJ, using the Infinicyt software program (Cytognos, Salamanca, Spain). Bone marrow samples from 10 healthy individuals, donors for hematopoietic stem cell transplantation (HSCT), were used as a control group for apoptosis experiments. The controls included 5 healthy pediatric donors and 5 healthy adult donors. This study was

reviewed and approved by the Ethics Committees of the National Cancer Institute (CEP#3401739), the Arthur Siqueira Cavalcanti Hematology Institute (HEMORIO) (CEP#063/05) and the IPPMG-UFRJ (CEP#08926213.9.0000.5264), and was conducted in conformity with the Declaration of Helsinki.

Apoptosis analysis by flow cytometry

To determine the percentage of apoptotic cells, we used fresh bone marrow cells from MDS patients and healthy individuals. Initially, it was used red blood lysis solution (RBC) for 5 minutes. After centrifugation, the supernatant was withdrawn. The bone marrow cells were washed in phosphate-buffered saline (PBS). Next, cells were divided into two aliquots ($1x10^6$) and stained with two different antibody combinations: CD34-PE/CD45-PerCP and CD14-PE/CD45-PerCP (BD Biosciences) for 15 minutes in the dark. In the following step, PBS was added and cells were centrifuged during 5 minutes. Finally, the cells were incubated with annexin-V-FITC and propidium iodide (PI) (Apoptosis Detection Kit II, BD Biosciences) for 20 minutes at room temperature protected from light, according to manufacturer's instructions. A total of 200,000 events were acquired using a FACSCalibur Flow Cytometer (Becton Dickinson, USA) and analyzed using the Infinicyt software program (Cytognos, Salamanca, Spain).

Cytogenetic study

Karyotypes of bone marrow cells were obtained from cultures in RPMI 1640, with 20% fetal calf serum (GIBCO) at 37 °C for 24 hours. Cell cultures were pulsed with colcemid to a final concentration of 0.05 µg/mL for the final hour of incubation. Cells were subsequently harvested by standard procedures (hypotonic shock: 0,075M) and fixed in methanol: acetic acid (3:1). GTG banding was performed. Chromosomes were identified and arranged according to the International System for Cytogenetic Nomenclature, 2016.[27]

Statistical analysis

The associations of the percentage of apoptosis between healthy individuals and MDS patients, patient age (adult and pediatric patients), bone marrow cellularity, disease subtype, cell populations (considering immature cells, $CD34^+$, and mature, $CD34^-$) and karyotypes were performed using the Mann-Whitney test. Our sample was considered statistically significant at $p<0.05$.

Results

Apoptosis analysis in bone marrow cells of primary MDS patients

The percentage of apoptotic cells in total BM cells from MDS patients (median: 9.9%; range 1.7%-55.4%, 81 patients) was significantly higher than the percentage from healthy BM donors (4.3%; 2.6%-5.7%, 10 individuals), $p<0.0001$ (Figure 1A). Considering age, rates of apoptosis in BM cells were similar in MDS adults (9.9%; range 1.7%-55.4%) and pediatric patients (9.5%; 2.9%-36%), $p<0.9$ (Figure 1B). Taking into account MDS BM cellularity, cases with hyper/normocellular BM had increased percentages of apoptotic cells (12.2%, range 1.7% to 55.4%) compared to healthy donors 4.3% (range 2.6%-5.7%), $p<0.0001$. Interestingly, MDS patients with hypocellular BM also had more apoptotic cells (8.6%; 2.9%-25.8%) than healthy donors, $p<0.0004$. Our results showed an increased rate of apoptosis in patients with MDS, independently of the cellularity of the BM. Furthermore, MDS patients with hyper/normocellular BM had a higher apoptosis rate than MDS cases with hypocellular BM ($p<0.01$) (Figure 1C).

Analysis of apoptosis and MDS subtypes

In our sample, of the 81 patients analyzed, 63 patients were classified as RA/RC, 15 as RAEB and 3 as RAEB-t. Analyzing the apoptosis according to each subtype of disease, it was observed that patients in early-stages (RA/RC) had significantly higher rates of apoptosis (11.3%; 2.8%-55.4%) than patients in more advanced stages (RAEB and RAEB-t), with a median percentage of apoptosis of 7.2% (range 1.7%-18.9%), $p<0.006$ (Figure 2A).

In patients in the early stages of the disease (RA/RC), cases with hyper/normocellular BM had a higher median percentage of apoptosis (14.2%; range 2.8%-55.4%) than patients with hypocellular BM (8.6%; range 3.2%-25.8%), ($p<0.007$) (Figure 2B). In the later stages of the disease (RAEB and RAEB-t), patients with hyper/normocellular BM had a similar median percentage of apoptosis (6.5%; range 1.7%-18.9%) to hypocellular BM cases (7.8% apoptosis, range 2.9%-13.6%), $p<0.9$ (Figure 2C).

Apoptosis on bone marrow hematopoietic progenitor cells ($CD34^+$cells) and bone marrow cells committed to differentiate lineages ($CD34^-$ cells)

An apoptosis analysis was performed on different cell populations to determine whether the pluripotent

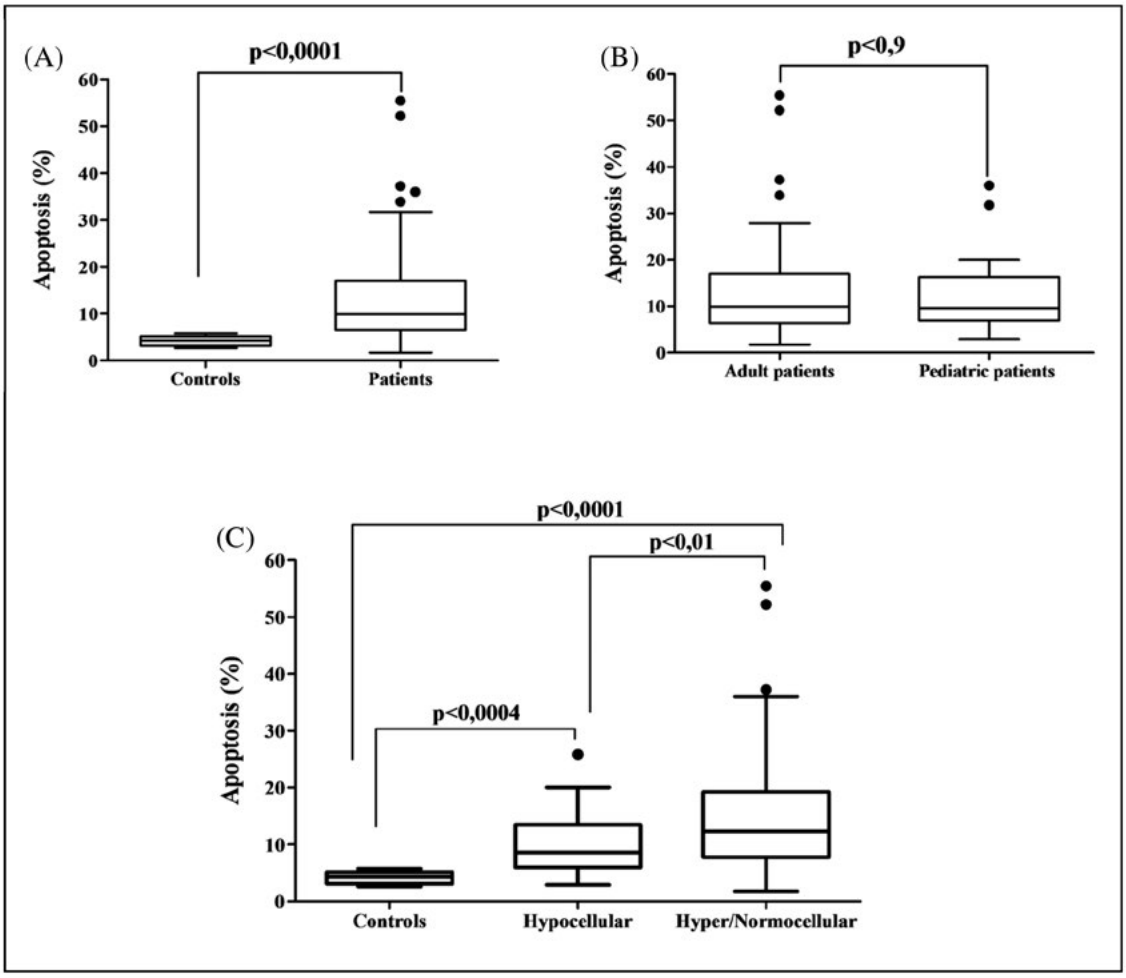

Figure 1. (A) Percentage of apoptotic cells: controls versus MDS patients; (B) Percentage of apoptotic cells: adults MDS patients versus pediatric MDS patients. (C) Percentage of apoptotic cells: controls versus hypocellular BM, controls versus hyper/normocellular BM, hypocellular BM versus hyper/normocellular BM. The results are shown in box-plot graphics in linear scale, showing the median, range, and the dots represent outliers

Source: The authors (2022).

stem cells ($CD34^+$ cells) or the cells already committed to differentiation were in apoptosis. This analysis showed that apoptosis occurs in $CD34^+$ cells. However, the percentage of apoptosis is higher in cells already involved in a cell differentiation program. This can be seen in the healthy individuals and patients, independent of BM cellularity. We observed that more mature cells had a median percentage value of apoptosis equal to 9.6% (range 1.7%-53.6%), while progenitor cells ($CD34^+$) showed a median percentage of apoptosis equal to 0.14% (range 0%-1.9%). Thus, the cells already committed to the program of cell differentiation had a higher percentage of apoptosis ($p<0.0001$) (Figure 3).

We used flow cytometry to analyze the percentage of apoptosis in specific hematopoietic cell populations according to the cellularity of bone marrow. We observed that lymphocytes are the cells with the lowest percentage of apoptosis compared with other hematopoietic cell populations, with median percentage value of apoptosis equal to 5.47% (range 0.37%-50.03%) in cases of hypocellular BM and 10.7% (range 0%-46.96%) in hyper/normocellular BM. Nucleated red blood cells (NRBC), granulocytic and monocytic cells showed higher apoptosis rates compared with both $CD34^+$ cells and lymphocytes. NRBC showed a median percentage value of apoptosis

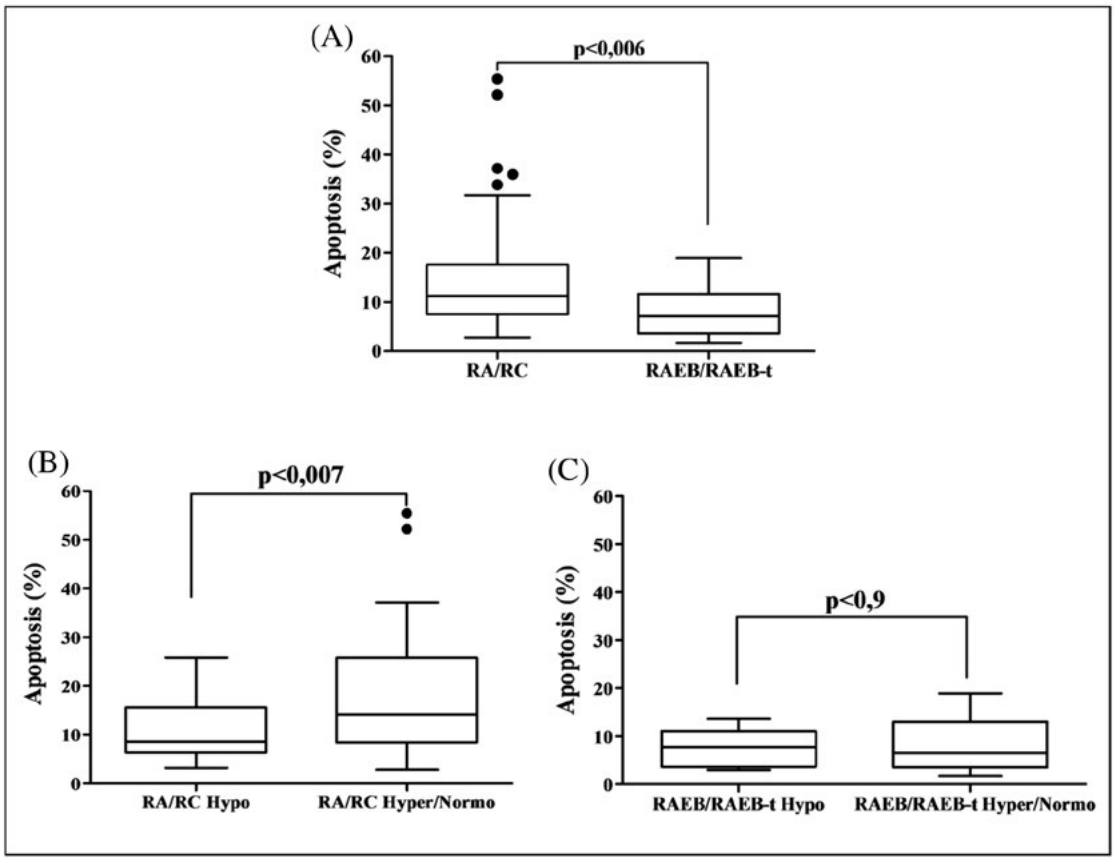

Figure 2. Analysis of apoptosis in bone marrow cells in different MDS subtypes. (A) Percentage of apoptosis: early stages (RA/RC) versus advanced stages (RAEB and RAEB-t). (B) Percentage of apoptosis: RA/RC Hypocellular BM versus RA/RC hyper/normocellular BM. (C) Percentage of apoptosis RAEB/RAEB-t Hypocellular BM versus RAEB/RAEB-t hyper/normocellular BM. The results are shown in box-plot graphics in linear scale, showing the median, range, and the dots represent outliers

Source: The authors (2022).

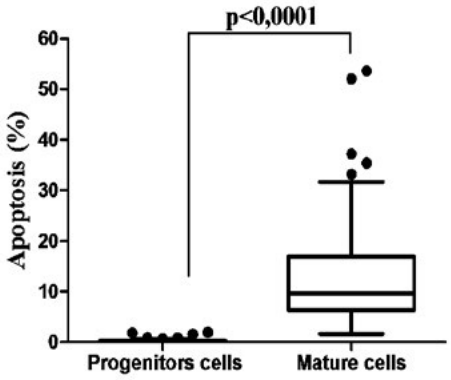

Figure 3. Percentage of apoptosis in progenitor cells versus mature cells from MDS patients. The results are shown in box-plot graphic in linear scale, showing the median, range, and the dots represent outliers

Source: The authors (2022).

of 43.1% (range 2.26%-87.17%) in hypocellular BM and 28.6% (range 0-91%) in hyper/normocellular BM. The granulocyte population had a median percentage value of apoptosis of 8.24% (range 3%-71.8%) in hypocellular BM and a median percentage value of apoptosis 12% (range 1.45%-90.71%) in hyper/normocellular BM. Monocytic populations showed a median percentage value of apoptosis of 24% (range 2%-100%) and 50.33% (range 1.78%-95.9%) in hypocellular and hyper/normocellular BM, respectively (Figure 4).

Comparison of the percentage of apoptotic bone marrow cells according to karyotypes

The analysis of the percentage of apoptosis and the karyotypes showed: the median percentage of apoptosis in normal karyotypes (n=34) was 9.1% (range 1.7%-55.4%); the median percentage of apoptosis in del(5q) (n=5) was 16.9% (range 2%-19.3%); in the case of patients with del(11q) (n=4) the median percentage of apoptosis was 6.4% (range 3.2%-8.2%); the del(17p) (n=8) had a median percentage of apoptosis of 12% (range 2.9%-15.6%) and patients with complex karyotypes (n=3) presented a median percentage of apoptosis of 3.7% (range 2.8%-4.9%). Patients with normal karyotypes, del(5q) and del(17p), presented higher apoptosis rates. Patients with del(11q) and complex karyotypes showed a decrease in apoptosis (Figure 5). The comparison of apoptosis in these two karyotype groups was statistically significant, $p<0.004$.

Discussion

Apoptosis has been presented as part of primary MDS pathogenesis.[28-30] Although many studies focus on MDS apoptosis, a review of the literature showed that little is known about apoptosis in pediatric primary MDS and about the difference in apoptosis between pediatric and adult patients.[9,28,31,32] In our study, we initially compared the presence of apoptosis in patients with primary MDS versus healthy individuals, which showed an increase of apoptosis in MDS patients. Then, we compared apoptosis rates in BM samples from adult and pediatric MDS patients, which were found to be similar, suggesting that MDS-related apoptosis is a process that is independent of the age. In relation to the apoptosis rate according to the BM cellularity in MDS patients, we observed a higher apoptosis in hyper/normocellular BM cases. Our results suggest that, despite having an increased percentage of apoptosis when compared to healthy individuals, the hypocellular BM of some MDS patients is probably not caused solely by apoptosis, and that other factors may be associated, such as, for example, alterations in the cell proliferation program of hematopoietic stem cells, in which the presence of a molecular alteration could induce silencing or decrease of the expression in one or more genes related to the cell proliferation program. The cause of hypocellular MDS is not completely understood. Serio and colleagues[33] reported that hypocellular MDS patients showed a severe deficit of immature hematopoietic progenitor cells, measured as secondary colony-forming cells (CFC), compared to healthy individuals, which would imply that immature hematopoietic stem cell compartment is affected by disease processes in hypocellular MDS. The damage to marrow hematopoietic progenitors occurring in hypocellular MDS may be explained by different immune-mediated mechanisms. Clinically, the strongest evidence for immune-mediated hematopoietic suppression in some hypocellular MDS is the response to immunosuppression, including mainly cyclosporine and anti-thymocyte globulin.

The high incidence of apoptosis is a remarkable feature observed in early stage of the MDS, while a decrease in apoptosis is observed in more advanced subtypes.[7,29] In our study, patients with RA/RC, independently of BM cellularity, showed a higher percentage of apoptosis when compared with patients with RAEB/RAEB-t. Some studies attributed the decrease in apoptosis rates of patients in advanced stages to increased levels of BCL2 protein and other anti-apoptotic proteins.[27,28] Increased expression of BCL2 protein has also been associated with increased resistance to apoptosis and leukemic transformation, and therefore a poor prognosis.[32]

The literature contains discussions on which cells would be entering in the apoptosis program, whether the progenitor cells or cells already committed to a cell differentiation program.[7,29] In our study, we observed a significant difference between apoptosis in cells already committed to a cell differentiation program and progenitor cells. Our results suggest that apoptosis is more intense in cells already committed to a cell differentiation program, maybe as an attempt of patients' own BM to remove dysplastic cells at beginning of the disease, as cited by Corey and colleagues.[7] According to Raza and colleagues[23] the immature $CD34^+$ cells are stimulated to proliferate, while their later differentiated daughters are induced to undergo apoptosis accounting for the clinical syndrome of pancytopenia.

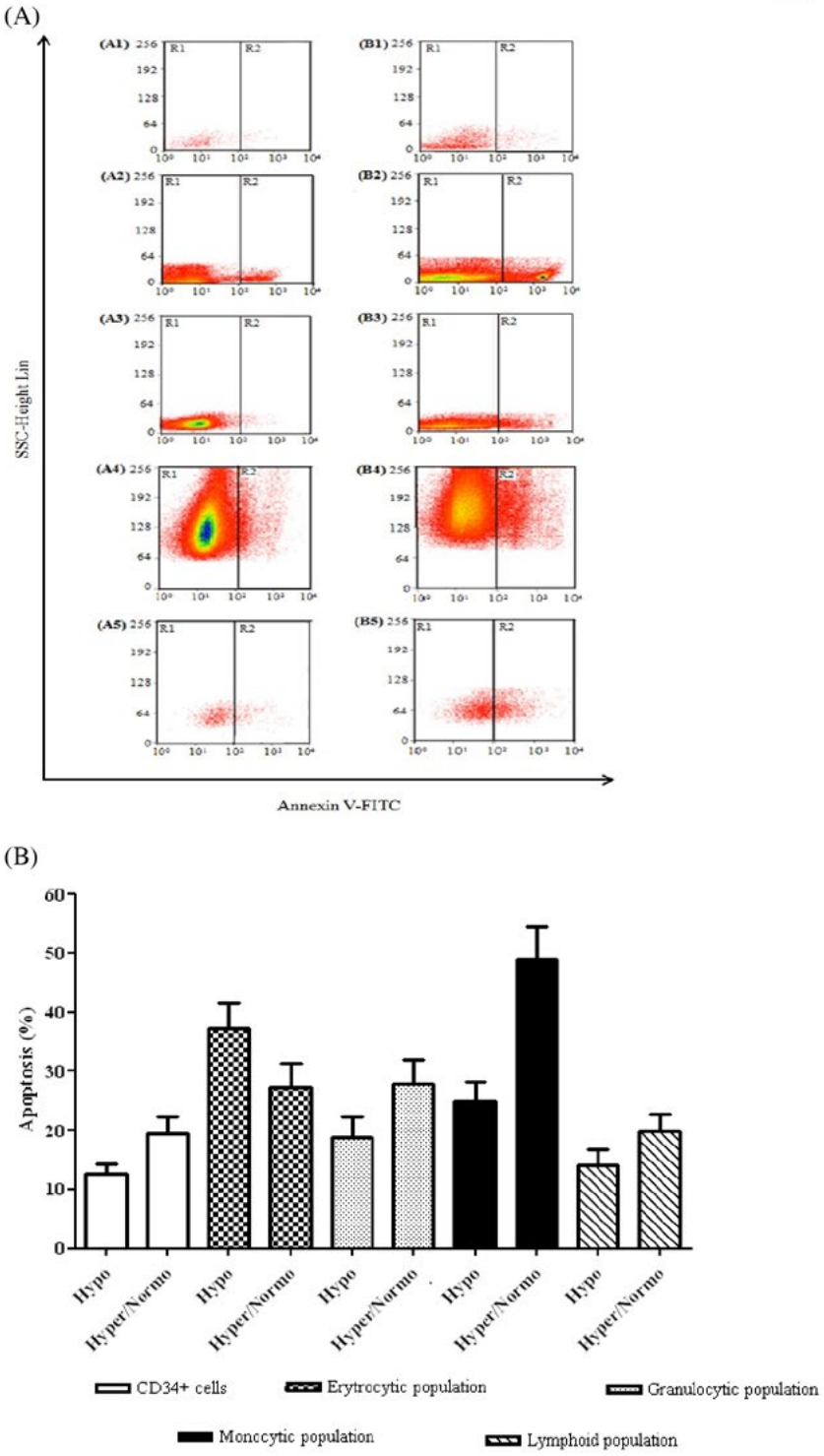

Figure 4. (A) Apoptosis analysis in specific hematopoietic cell populations in MDS patients by flow cytometry. In column A, sample of hypocellular BM MDS and in column B, sample of hypercellular BM MIDS. Apoptotic cells are in gate R2. Al and B1 are CD34* cells; A2 and B2 erytrocytic population; A3 and B3 lymphoid population; A4 and B4 granulocytic population; A5 and B5 monocytic population. (B) Comparison of apoptosis between specific hematopoietic cell populations in BM of hypocellular and hyper/normocellular MDS patients

Source: The authors (2022).

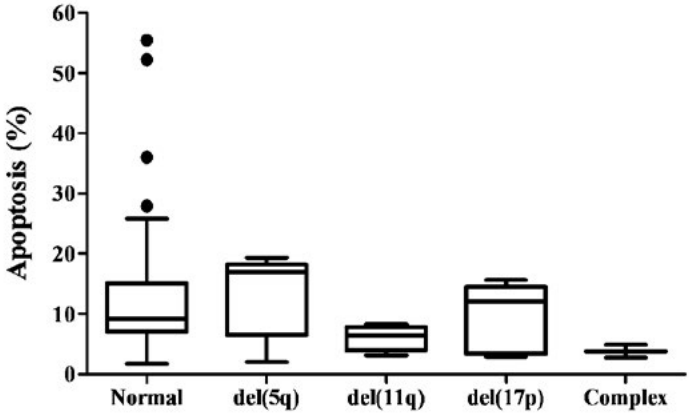

Figure 5. Association between the percentage of apoptosis and the karyotype in MDS patients. The results are shown in box-plot graphic in linear scale, showing the median, range, and the dots represent outliers

Source: The authors (2022).

Few studies have been conducted to try to identify an association between apoptosis and karyotypes of MDS patients. Washington and colleagues[20] and Sloand and colleagues[21] associated the presence of del(5q) and trisomy 8 with a reduction in the rate of apoptosis in MDS. In our study, MDS patients with normal karyotypes, del(5q) and del(17p) had significantly higher apoptosis rates than MDS cases with del(11q) and complex karyotypes.

An intriguing question related to apoptosis in MDS is a possible association with the paradox of the disease (BM usually hypercellular with peripheral blood cytopenias), in which apoptosis was the cause of ineffective hematopoiesis[8,34,35] or apoptosis would be related to the paradigm of the disease, where the presence of dysplasias (failure of maturation of hematopoietic cells) would lead to apoptosis as a physiological process, where the hematopoietic system would be trying to eliminate clones with dysplastic defects in early stages of the disease.[10,36,37] Thus, in this latter case, defects in differentiation or in maturation would be the cause of ineffective hematopoiesis, not apoptosis. Our results suggest that apoptosis may be a consequence of inefficient hematopoiesis. Therefore, apoptosis in primary MDS is related to the disease paradigm. In addition, we suggest that apoptosis is not the main mechanism associated with hypocellular MDS, and occurs preferentially in primary MDS cases of hyper/normocellular that are associated with a good prognosis. To our knowledge, this is the first study analyzing the percentage of apoptosis in BM of patients with primary MDS and their associations with patient age, BM cellularity and different karyotypic patterns.

Conclusions

The BM cells committed to differentiate ($CD34^-$ cells) had a higher percentage of apoptosis than BM hematopoietic progenitor cells ($CD34^+$ cells). Patients with normal karyotypes, del(5q) and del(17p) had higher apoptosis rates in comparison to patients with del(11q) and complex karyotypes. Apoptosis is an important characteristic of BM cells in adult and pediatric MDS patients, where the hematopoietic system would be trying to eliminate cells with dysplastic defects in the early stage of the disease.

Potential conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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