Immunophenotypic analysis of myelodysplastic syndromes

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Background and Objectives. In contrast with hematologic malignancies in which the value of immunophenotypic studies is well established, information on the immunophenotypic characteristics of myelodysplastic syndromes (MDS) is scanty. The main goal of the present study was to explore the immunophenotypic differences between patients with MDS and normal individuals, including changes in distribution of cell lineages as well as phenotypic aberrations and blockades in cell maturation pathways.

Design and Methods.In MDS the proportion of bone marrow $CD34^+$ cells was higher than in normal patients but the most immature progenitors ($CD34^+CD38^-$) were less represented. By contrast the proportion of myelomonocytic $CD34^+$ cells was greater than in normal individuals, translating into an increased myeloid/non-myeloid $CD34^+$ hematopoietic progenitor cell ratio.

Results. This suggests that in MDS, the majority of $CD34^+$ cells are already committed to the myeloid lineage. Upon analyzing the granulo-monocytic differentiation pathway, MDS patients showed an increased proportion of monocytic cells with a decreased percentage of cells of neutrophil lineage, leading to a lower neutrophil/monocytic cell ratio. Maturational arrests in the monocytic but not in the neutrophil differentiation pathway were observed. In refractory anemia with excess blasts in transformation (RAEB-t) such blockades mainly occurred during the earliest stages of differentiation but in the other MDS subtypes they occurred in later stages.

Interpretation and Conclusions. Phenotypic aberrations occurred in 90% of patients and a high proportion of cases showed $\geq$2 aberrations. In summary, our results show that, in addition to an abnormal distribution of the bone marrow cell compartment, MDS patients frequently show aberrant phenotypes and maturational arrests. Some of these features may help in cases in which the diagnosis of MDS is questionable.

Key words: immunophenotype, myelodysplastic syndromes, phenotypic aberrations, differentiation blockades.

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Myelodysplastic syndromes (MDS) are a group of clonal stem cell disorders characterized by ineffective hematopoiesis which leads to quantitative and qualitative cell abnormalities with one or more peripheral blood (PB) cytopenias, and an increased risk of progression to acute myeloid leukemia (AML).

In contrast to other hematologic malignancies in which the value of immunophenotypic studies is well established,[1-4] information on the immunophenotypic characteristics of MDS is scanty. This is probably related to the fact that in MDS patients, the pathologic cell clone is heterogeneous and matures into different cell lineages associated with dysplastic features. Most reported studies have been based on the analysis of single antigen stainings and have shown the presence of frequent abnormalities in expression (increased or decreased) of different antigens.[5-8] More recent studies suggest that dysplastic features of the clonal bone marrow (BM) cells from MDS patients might translate into abnormal immunophenotypic profiles,[9] but the clinical impact of these phenotypic findings still needs to be established.

In the present study we have investigated the immunophenotypic features of BM cells in a large group of MDS patients using multiparametric flow cytometry. The main goals of the study were: 1) to explore the immunophenotypic differences between MDS and normal BM including 2) the changes in distribution of different cell lineages, and 3) phenotypic aberrations and blockades in cell maturation pathways.

Design and Methods

Patients and Controls

A total of 101 patients, diagnosed with MDS according to the WHO criteria,[10] were included in this study. The distribution of these patients according to the final diagnosis was as follows: refractory anemia (RA) (21 cases); RA with ringed sideroblasts (RARS) (20 cases); RA with excess blasts (RAEB) (9 cases); MDS with multilineage dysplasia, (16 cases) and MDS/myeloproliferative disease (35 cases). Cases with chronic myelomonocytic leukemia (CMML), classified according FAB criteria were included (n=30). In addition 13 patients with RAEB in transformation (RAEB-t), diagnosed according to the FAB classification,[1] were also included in the study. Patients were reclassified retrospectively after prior FAB classification had been performed. Cases not fulfilling WHO criteria were not included in the analysis. In all cases, flow cytometry immunophe-

notypic studies were performed on BM samples obtained at diagnosis. The median age of the MDS patients was 74 years (range: 13 to 91); only one child was included in this series. Sixty-nine were males and 45 females. By the end of the study 19 patients had developed a secondary AML (sAML) within a median of 13 months (range 1 to 42 months). Median overall survival (OS) in the whole series was 30 months (95% confidence interval of 19 to 41 months) and 36 deaths had been registered.

Twelve BM samples from healthy adult volunteers undergoing orthopedic surgery at the University Hospital of Salamanca and who had given informed consent were used as controls. The median age of this control group of healthy volunteers was 45 years (range: 20 to 72 years), with seven (58%) males and 5 (42%) females. BM samples from both the MDS patients and the controls were obtained from the sternum or posterior iliac crest and placed in tubes containing heparin as anticoagulant until being processed for immunophenotypic analyses.

Immunophenotypic studies

Whole BM samples (approximately 2×10^6 cells in 100 μL/test) were stained using a stain-and-then-lyse direct immunofluorescence technique in which the following monoclonal antibodies (MoAb) were used in triple-stainings-fluorescein isothiocyanate (FITC), phycoerythrin (PE), and PE cyanine5 (Cy5)-: CD34/CD33/CD38, CD15/CD34/HLADR and HLADR/CD13/CD45. Briefly, BM samples were incubated (15 min at room temperature), in the presence of 5-20 μL of each MoAb, according to the recommendations of the manufacturers. After lysing the non-nucleated red cells with 2 mL/tube of FACS lysing solution (Becton Dickinson Biosciencies, San José, CA, USA), cells were centrifuged (5 min at 540 g) in phosphate-buffered saline (PBS) and resuspended in 0.5 mL of PBS/tube until analyzed in the flow cytometer.[11] All MoAb reagents were purchased from Becton Dickinson Biosciences (BDB) except CD38-PE Cy5 and CD45-PE Cy5 which were obtained from Caltag Laboratories (San Francisco, CA, USA). Data acquisition was performed in two consecutive steps on a FACScalibur (BDB) flow cytometer using CellQUEST software (BDB). In the first step, a total of 20,000 events/tube were acquired, corresponding to the total nucleated BM cells. In the second step, acquisition through electronic *live-gates* drawn on $CD34^+$ cells and/or $HLADR^+$ cells was performed according to the cells' SSC and antigenic expression (Figure 1A). In this latter step, 3×10^5 events were measured with information only obtained for those events that fulfilled the live-gate criteria. The Paint-A-Gate PRO software program (BDB) was used for data analysis.

The following BM cell compartments were analyzed for immunophenotype: 1) $CD34^+$ hemopoietic stem and progenitor cells (HPC), 2) neutrophil lineage cells ($CD45^+/SSC^{int/hi}$), 3) monocytic cells ($CD45^+/^{++}/SSC^{lo/int}$), 4) erythroid precursors ($CD45^{-/+}$ SSC^{lo}) and 5) lymphoid cells ($CD45^{++}/SSC^{lo}$). Erythroblasts were analyzed based on a FSC/SSC gate after excluding platelets and cell debris according to previously established methods.[12] In addition, further subsetting of several of these cell populations was performed. Accordingly, within $CD34^+$ HPC, the following cell subsets were specifically identified: $CD33^-/CD38^-$, $CD33^+/CD38^-$, $CD33^+/CD38^+$, $CD33^-/CD38^+$, $CD15^-/HLADR^-$, $CD15^-/HLADR^+$ and $CD15^+/HLADR^+$ cells. Furthermore, among cells from the neutrophil lineage, the mature and immature compartments were identified on the basis of CD13 and HLADR expression ($CD13^{++}/HLADR^-$ vs $CD13^+/HLADR^{+/-}$). Finally, up to 6 different stages of maturation were also defined for BM monocytic lineage cells after gating on intermediate $SSC/HLADR^+$ events from the earliest non-committed myeloid precursors to mature monocytes: $CD34^{++}/CD15^-$; $CD34^{++}/CD15^+$; $CD34^{++}/CD15^{++}$; $CD34^+CD15^{++}$; $CD34^-/CD15^{++}$; $CD34^-/CD15^{+/-}$ as has been previously reported.[13]

Those antigenic profiles present in the BM from some MDS patients but which were constantly undetectable in BM samples from healthy volunteers were considered as aberrant phenotypes. Overall, with the triple-stainings used, four different phenotypic aberrations were identified: 1) $CD34^+/CD15^+/HLADR^-$ cells; 2) CD33 over-expression on mature ($SSC^{hi}/CD45^{+/+}$) neutrophils; 3) HLADR expression on mature ($SSC^{hi}/CD45^{+/+}$) neutrophils; and 4) abnormally low CD45 expression on $SSC^{int}/CD15^-/HLADR^{++}$ cells.

Statistical analysis

For all variables under study, the frequency or mean value ± standard deviation, median and range were calculated using the SPSS 8.0 software program (Chicago, USA). The statistical significance of the differences observed between groups was assessed using either parametric (Student's t test) or non-parametric (Mann-Whitney U) tests.

A correlation was performed using Spearman's test in order to ascertain whether there was a relationship between the percentage of BM blast cells and the $CD34^+$ cell proportion.

Results

Immunophenotypic characteristics of BM cells in MDS

Table 1 shows the distribution of the major cell subsets present in BM samples from MDS patients

Table 1. Distribution of different cell populations in BM samples from MDS patients and healthy controls.

% of cells	*MDS patients n. 114*	*Controls n. 12*	*p*
CD34+*	3.6±5.5	1±0.3	<0.001
Monocytic cells*	9.2±7	5.3±2	<0.001
Neutrophil lineage*	49±17	70.5±10	0.001
Neutrophil/monocytic cell ratio	8.3±6.2	18±8.5	0.002
Mature neutrophils°	51.2±21.5	52.5±6.6	0.8
Immature neutrophils°	42±21.4	48±6.6	0.1
Mature/immature neutrophil ratio	5±13	1.1±0.3	0.4
Red cells*	21.1±16	8±5.3	<0.001
Lymphocytes*	11±9.2	12.1±5	0.7

**Results are expressed as mean ± standard deviation of the percentage of the total BM nucleated cells.*
°Results expressed as mean ± standard deviation of the percentage of BM cells within the neutrophil lineage.

Table 2. Distribution of different CD34⁺ cell subsets in BM samples from MDS patients and healthy controls.

CD34⁺ HPC subset	*MDS n. 114*	*Controls n. 12*	*p value*
$CD34^+/CD38^+/CD33^-$	18±20.5	40±25	0.002
$CD34^+/CD38^+/CD33^+$	56±30	29±19	0.01
$CD34^+/CD38^-/CD33^+$	16.2±24	16±23	0.8
$CD34^+/CD33^+/CD34^+CD33^-$ cell ratio	40±81	1.2±1.6	<0.001
$CD34^+/CD15^-/DR^-$	17.7±20.4	22.712	0.4
$CD34^+/CD15^-/DR^+$	58±27.4	65.2±10	0.09
$CD34^+/CD15^+/DR^+$	21±38	11.6±3	0.4

Results expressed as mean±standard deviation of the percentage of cells from total CD34⁺ HPC.

and healthy controls. As can be seen, compared to normal BM, the BM from patients with MDS showed higher percentages of $CD34^+$ HPC (3.6±5.5% vs 1±0.3%, p <0.001) monocytic (9.2±7% vs 5.3±2%, p <0.001) and red cells (21 ±16% vs 8±5.3 %, p <0.001) together with lower proportions of cells from the neutrophil lineage (49±17% vs 70.5±10%, p=0.001). This was associated with a decreased neutrophil/monocytic cell ratio (8.3±6.2% vs 18±8.5%, p=0.002) among MDS cases. In contrast, no major differences were observed between MDS and normal BM as regards the distribution of lymphocytes and the relative proportion of $CD13^{++}$ (mature) and $CD13^+$ (immature) cells within the neutrophil lineage (51.2±21.5 vs 52.5±6.6, p=0.8 and 44±21.4 vs 48±6.6, p=0.1, respectively).

Regarding $CD34^+$ HPC, it should be noted that the overall number of these cells showed a good correlation with the number of BM blast cells assessed by morphology (r= 0.76; p<0.001). Interestingly, upon analyzing the $CD34^+$ cell subsets (Table 2) the proportion of the most immature subpopulation ($CD34^+CD38^-$) was similar in MDS patients and controls; conversely, the myeloid-committed ($CD34^+CD33^+$) progenitors were more numerous in MDS patients, leading to a significantly higher $CD34^+$ $CD33^+/CD34^+CD33^-$HPC ratio in MDS patients as compared to among controls (p<0.001) (Table 2). As regards the distribution of the three cell subsets defined on the basis of the combined expression of the CD15 and HLADR antigens on $CD34^+$ cells, no major differences were observed between MDS and controls (Table 2).

Overall, the presence of phenotypic aberrations (defined in *Design and Methods*) were detected in about 90% of all MDS cases analyzed. Of the aberrations observed the most frequent was the presence of cells displaying a $CD34^+CD15^+HLADR^-$ phenotype (73% of the cases) while the least frequent was an abnormally low CD45 expression on monocytes (5%). Overexpression of CD33 on neutrophils and aberrant HLADR expression on mature granulocytes were found in 71% and 27% of the cases, respectively (Table 4).

Interestingly, maturation blockades were not observed in the neutrophil lineage. By contrast, MDS patients frequently (67%) showed some degree of maturational arrest within the cells from monocytic lineage. In the majority of cases such blockades occurred in the final stages of maturation and cells only accumulated in the initial stages of maturation in 10% of the patients analyzed (Figure 1, Table 3).

Phenotypic differences according to MDS subtype

Upon grouping the MDS patients according to the different disease subtypes we observed that, as might be expected, patients with RAEB and RAEB-t had the highest proportions of $CD34^+$ (Figure 2A). Despite this, the distribution of the different $CD34^+$ cell subsets was similar in all MDS subtypes, except for RARS and MDS-md which displayed a higher frequency of immature precursors ($CD34^+/CD38^-/CD33^-$) with values similar to that observed in normal BM (Figure 2B). As mentioned above, the proportion of monocytic cells

Table 3. Maturational arrest within BM cells from the monocytic compartment according to the MDS subtype.

	Stage of maturational arrest			
	% of cases with maturation arrest	Early (1&2)	Intermediate (3)	Late (4&5)
MDS-md (n=16)	80	1	0	11
RA (n=21)	74	0	0	14
RARS (n=20)	58	1	0	10
RAEB (n=8)	37	0	0	3
RAEB-t (n=11)	100	5	0	6
MDS/MPD (n=35)	71	3	1	20
Total (n=111)	67	10	1	64

Results are expressed as number of cases showing a maturational arrest. See design and methods for the specific determination of each maturational stage.

Table 4. Incidence of abnormal phenotypes according to the MDS subtype.

	Type of aberrant phenotypes			
% of aberrant cases*	$CD15^+$ HLA^- DR^-CD34^+HPC	CD45dim monocytes	CD33 overex.	HLA^-DR^+ neutroph.
MDS-md 88 (n=16)	69	0	56	6
RA 100 (n=21)	58	5	84	21
RARS (n=20) 90	89	0	47	6
RAEB (n=9) 100	88	37	100	50
RAEB-t (n=13) 85	73	36	60	9
MDS/MPD(n=35) 94	71	3	74	42
Overall(n=114) 94 frequency	73	5	71	27

Results expressed as percentage of cases within each subtype with one or more phenotypic aberration.

was significantly increased in the overall population of MDS patients. When these results were analyzed according to the MDS subtype, a higher proportion of monocytic cells was detected among MDS/MPD cases followed by RAEB patients (Figure 3B), which resulted in a lower neutrophil/monocytic cell ratio in these two subgroups. In contrast, RAEB and RAEB-t cases showed a higher proportion of mature neutrophils (Figure 3E).

Regarding phenotypic aberrations, the overall incidence was similar in all MDS subgroups. Nevertheless, the coexistence of ≥ 3 phenotypic aberrations was more common in RAEB patients. Moreover, low CD45 expression on monocytes was significantly more frequent in RAEB and RAEB-t patients (Table 4).

Finally, the frequency of blockades in the monocytic differentiation pathway ranged between 37% in RAEB patients up to 100% in RAEB-t cases (Table 3).

Discussion

Until now, few systematic immunophenotypic studies have been performed comparing the distribution of the different cell lineages in MDS and in normal BM. Moreover, there is very little information on the potential diagnostic and prognostic value of immunophenotyping in MDS. Recently, Stetler-Stevenson *et al.*[9] reported on the utility of flow cytometry immunophenotyping in the diagnosis of MDS based on abnormalities in the phenotypic features shown by the different cell compartments investigated. Although they did not recommend their approach as a screening procedure, they did suggest that it may help in cases in which the diagnosis proves difficult.

One difficult decision in these studies is how to choose controls since MDS cases are mostly observed in old patients. Our control cases are younger and their pathologies do not influence hematopoiesis (orthopedic diseases). Older patients most frequently display bone fractures which may stress the BM.

Most information available on the value of immunophenotypic studies in MDS relates to the analysis of $CD34^+$ HPC.[6,14,15] In the present study, as a first step, we analyzed the $CD34^+$ hematopoietic progenitor cell compartment and its subsets. As was previously shown Guyotat *et al.*,[6] the proportion of $CD34^+$ cells was higher in MDS BM than in control BM, and the proportion of $CD34^+$ cells correlated with the percentage of BM blast cells identified by morphology.[6,14,16] It has also been reported that increased CD34 expression is associated with a poor IPSS and a high blast cell count.[17] Regarding the distribution of $CD34^+$ cell subsets in relative numbers, the most immature progenitors ($CD34^+CD38^-$) were less represented in the BM of MDS patients. In contrast, the proportion of myelo-monocytic $CD34^+$ cells was significantly higher than that in normal individuals, translating into an increased myeloid/non-myeloid $CD34^+$ HPC ratio.

This suggests that the majority of $CD34^+$ cells in MDS are already committed to the myeloid lineage. Interestingly, upon analyzing the different $CD34^+$ cell subsets according to the subtype of MDS, we observed that only MDS-md patients showed a normal proportion of $CD34^+$ cells together with a normal $CD34^+CD33^+/CD34^+CD33^-$ cell ratio. These findings suggest that a normal hemopoietic pattern is better preserved in the MDS-md subtype.

Since the WHO classification has only recently been applied[10] there are no other references to compare with these observations.

Upon analyzing the granulomonocytic differentiation pathway, it became evident that MDS patients had an increased proportion of monocytic cells and a decreased percentage of cells from the neutrophil lineage, leading to a lower neutrophil/monocytic cell ratio. These features are consistent with the results of Mittelman *et al.*, who suggested that among MDS BM cells, the HLA-DR subpopulation is increased,[8] and support the hypothesis that the monocytic component may be more relevant in the pathogenesis of MDS than previously suspected.[18]

Since MDS are considered to represent preleukemic processes, we investigated the presence of two frequent features of AML: maturational arrests in the differentiation pathway and phenotypic aberrations. Surprisingly, with the combinations of monoclonal antibodies used, we observed maturational arrests in the monocytic but not in the neutrophil differentiation pathway. In RAEB-t, such maturational arrests mainly occurred during the earliest stages of differentiation, demonstrating that this MDS subtype is close to AML or even, as proposed by the WHO classification, a diagnosis of sAML should be made. By contrast, in the other MDS subtypes, the blockades occurred in later stages of maturation.

Phenotypic aberration is a common characteristic of AML patients.[11] In the present study, four phenotypic aberrations were identified: the existence of $CD34^{+}CD15^{+}HLADR^{-}$ cell populations, dim CD45 expression on monocytic cells, overexpression of the CD33 antigen on cells from the neutrophil lineage and reactivity for HLADR on relatively mature neutrophils. The differences between RAEB and RAEB-t according to HLADR antigen were striking. Perhaps this could be explained by the lower proportion of mature neutrophils among RAEB-t cases. Interestingly, a high proportion of cases showed two or more phenotypic aberrations, which might be a consequence of the dyshematopoietic process. In addition, some of these aberrations, such as low CD45 expression on monocytic cells, were significantly more common in RAEB and RAEB-t patients, which suggests that they may warn of progressive disease. Moreover, $CD45^{dim}$ expression could help to discriminate RAEB and RAEB-t from other MDS subtypes.

In summary, our results show that, in addition to an abnormal distribution of the BM cell compartment, MDS patients frequently show aberrant phenotypes and maturational arrests, some of which may help in cases in which the diagnosis of MDS is questionable.

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Contributions

CdC designed and supervised the study. She wrote the manuscript. EF, AL, BV and EV performed the immunophenotypic studies. JLA and FO recruited the patients and fulfilled alla databases. EF and JLA analyzed data. AO and JFSM supervised the cytometry analisis and critically revised the manuscript.

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Disclosures

Conflict of interest: none.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Marie-Christine Béné, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr Béné and the Editors. Manuscript received July 9, 2002; accepted February 26, 2003.

In the following paragraphs, the Associate Editor summarizes the peer-review process and its outcomes.

What is already known on this topic

Only few studies have been published so far on immunophenotyping in myelodysplasia, and controversial conclusions were drawn from them. This topic has gained interest recently and more positive results have been reported stressing the interest of this technique.

What this study adds

Here, a comparison between normal and dysplastic marrow was performed, demonstrating differences in $CD34^+$ compartments, as well as in monocytic and neutrophil lineages, which may be useful for the detection of MDS.