Journal of BUON **15:** 746-752, 2010

ORIGINAL ARTICLE

Clinical implications of immunophenotypic abnormalities of bone marrow myeloid cell compartment in myelodysplastic syndromes

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Summary

Purpose: *To evaluate the biological and clinical implications of immunophenotypic abnormalities of bone marrow myeloid cell compartment in patients with primary myelodysplastic syndromes (MDS).*

Methods: *Analysis of cell surface antigen profiles was performed by flow cytometric immunophenotyping on bone marrow mononuclear cell (BMMNCs) specimens from 39 adult MDS patients and 5 healthy individuals. Expression of cell surface antigen profiles was correlated with FAB subtype, cytogenetics, CFU-GM colony growth, overall survival (OS) and leukemic transformation (LT).*

Results: *Expression levels of early differentiation and myelo-monocytic antigens (CD38, CD13, CD33, CD14 and CD15) on myeloid cell compartment of BMMCs were significantly higher in MDS patients in comparison to healthy control group, suggesting maturational left shift of bone marrow myeloid cell compartment in MDS. CD34 antigen expression was in a positive linear correlation with HLA-DR antigen expression ($r=0.652$; $p=0.0004$), and in negative correlation with the expression of CD11b and CD15 antigens ($r=-0.48$; $p=0.014$ and $r=-0.564$; $p=0.0033$, respectively). Myeloid antigen ratio (HLA-DR/CD11b) was 2.5 fold higher in patients with MDS in comparison to control group. Patients with advanced disease had significantly higher myeloid antigen ratio than patients with low risk MDS ($p<0.05$). The type of CFU-GM colony growth and the presence of chromosomal aberrations were unrelated to the proportion of $CD34^+$ cells and elevated myeloid ratio. Patients with elevated proportion of $CD34^+$ BMMNCs or elevated myeloid ratio had significantly shorter OS and higher LT rate in comparison to patients whose proportion of $CD34^+$ BMMNCs and myeloid ratio were within normal range.*

Conclusion: *The presence of abnormalities in antigen expression profiles of bone marrow myeloid cell compartment has clinical implication in MDS, with particular contribution in predicting the patient outcome. Elevated proportion of $CD34^+$ BMMNCs and elevated myeloid ratio of bone marrow myeloid compartment are useful immunophenotypic tools for estimation of prognosis in this very heterogeneous disorder group.*

Key words: bone marrow, flow cytometry, immunophenotyping, myelodysplastic syndromes, myeloid cells, prognosis

Introduction

For many types of hematologic neoplasias, immunophenotypic profiles of the neoplastic cell population provide valuable information regarding a diagnosis, classification and prognosis [1]. However, the clinical value of immunophenotypic data has not yet been firmly established for MDS [1-4]. MDS are clonal hematopoietic stem cell disorders associated with a variety of maturation and functional abnormalities of mature and maturing cells, many of them being connected to surface antigens expression abnormalities [4,5]. Surface antigens serve as biological sensors and signal transmitters allowing various cells to interact with different stimuli and perform different functions. Several mechanisms may be involved in the abnormal expression of surface antigens in primary MDS including defective granulopoiesis, defective intracellular storage pool, abnormal membrane of cytoplasmic granules, and the effect of high levels of marrow cytokines such as tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) [5].

Several surface antigens have shown abnormal expression in MDS, including increased, decreased or

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Received 21-02-2010; Accepted 30-03-2010

lineage-aberrant antigen expression [5]. Abnormalities of surface antigens may be also associated with defective cell function and may indicate a more severe or more advanced stage of the disease [5]. Increased proportion of bone marrow cells expressing early or immature markers, such as CD13, CD33, CD34 and HLA-DR, has been associated with poor outcome and progression to acute myeloid leukemia [4,6-12]. Immunophenotyping by multiparametric flow cytometry is an accurate method for quantitative and qualitative evaluation of hematopoietic cells, and several groups have used flow cytometry in the study of MDS [2-4,11-15]. However, there are numerous discrepancies and inconsistencies in the literature in reviewing surface marker patterns in MDS, particularly from the methodological standpoint. Various methods have been used to express abnormal results including proportion of positive or negative cells and mean fluorescent intensity [5]. Moreover, there are only a few studies dealing with direct correlation between immunophenotypic abnormalities of hematopoietic cells and clinical characteristic and prognosis of MDS patients [3,4,8,12,16].

We explored the presence of surface antigen abnormalities of BMMNC compartment in MDS patients, with the aim to estimate the biological and clinical implications of immunophenotypic data in these disorders.

Methods

Patients

Included were 39 adult patients with primary MDS, diagnozed according to the criteria of FAB Cooperative Group [16]. All patients met the "minimal diagnostic criteria" for MDS, recommended by Reizenstein and Ost [17]. Patients with secondary and therapy-related MDS were excluded. End points for evaluation were acute myeloid leukemia (AML) development, death, or the date of the last follow-up. The control group consisted of 5 healthy individuals (2 volunteers and 3 individuals with unexplained monocytopenia in whom blood counts spontaneously became normal during follow up).

Immunophenotyping

Cell surface membrane antigen analysis was performed on heparinized bone marrow specimens by using indirect immunofluorescence and flow cytometry methods (EPICS-C, Coulter® or FACScalibur, Becton Dickinson®) applied on BMMNCs, as previously described [18]. A panel of commercial mouse anti-human monoclonal antibodies against leukocyte antigens was as follows: CD34 (HPCA-1), HLA-DR (Anti-HLA-DR), CD11b (Leu-15), CD15 (Leu-M1), CD38 (Leu 17) and CD61 by Becton Dickinson; CD13 (My7), CD33 (My9), CD14 (My4) and CD41 (Plt 1) by Bekman Coulter; and Glycophorin A (BMA 0160) by Behring Diagnostics. GAM-FITC was used as a second step reagent. For all antigens results were recorded on bone marrow whole myeloid population, gated according to intermediate forward-light/orthogonal light scatter properties [19]. In case of CD34 and GpA antigens, results were recorded on total BMMNCs. Antigen expression was given as a proportion of labeled cells in a studied population and compared to antigen expression of the same population in the bone marrow of the control group (5 healthy individuals). Values over 95% confidence range of the control group for expression of each antigen were considered as abnormal (Table 1). To avoid conclusions on prognostic significance of a single antigen, we also used the "myeloid ratio", as a ratio between proportions of cells expressing "immature" (HLA-DR) and "mature" myeloid antigen (CD11b) [20]. For analysis, values of "myeloid ratio" (HLA-DR/CD11b) over 95% confidence range of the control group were considered as abnormal (Table 2).

Table 1. Immune phenotype of bone marrow mononuclear cells (BMMNCs) in 39 patients with MDS. Results are expressed as a percentage of labeled BMMNCs reactive with monoclonal antibody against particular antigen

Antigen	*MDS* $x \pm SEM$	*Control group** $x \pm SEM$	*p-value*
CD13	36.2 ± 3.8	9.0 ± 1.3 (4.7-13.3)	0.006
CD33	48.1 ± 4.8	14.0 ± 2.9 (4.7-23.3)	0.006
CD14	17.1 ± 2.9	4.7 ± 1.6 (1.2-11.3)	0.04
CD11b	68.8 ± 3.6	73.0 ± 2.6 (61.6-84.4)	NS
CD15	42.0 ± 5.6	16.7 ± 4.8 (5.2-33.2)	0.03
GpA	11.7 ± 3.0	7.3 ± 2.2 (2.1-16.7)	NS
CD41	11.4 ± 3.2	4.3 ± 1.3 (1.4-10.1)	NS
HLA-DR	26.1 ± 3.3	19.0 ± 1.73 (11.5-26.4)	NS
CD38	53.1 ± 8.3	18.3 ± 3.9 (8.4-28.3)	0.006
CD34	7.60 ± 1.8	4.00 ± 0.6 (1.5-6.5)	NS

*Values within 95% confidence range of control group are in parenthesis
SEM: standard error of the mean, NS: not significant

Table 2. Myeloid ratio (HLA-DR/CD11b) in the MDS group compared with the healthy control group

MDS $x \pm SEM$	*Control group** $x \pm SEM$	*p-value*
0.64 ± 0.21	0.26 ± 0.02 (0.16-0.36)	NS

*In parenthesis are values within 95% confidence range, SEM: standard error of the mean, NS: not significant

Cytogenetic studies

Cytogenetic analysis was performed at diagnosis on unstimulated bone marrow cells by direct preparation and following 24-h culture, in RPMI 1640 medium with 25% fetal calf serum at 37° C. The HG-banding method [21] was used for harvesting and preparation of the metaphase and interphase slides. The karyotype was interpreted according to the International System for Human Cytogenetic Nomenclature [22].

Colony assays

In vitro cell culture studies were done at referral, according to the method described by Iscove [23] and Messner [24]. A culture containing less than two colonies (CFU-GM) and/or less than 20 GM clusters was considered a "no growth". A culture containing low colony and high cluster number or normal/high colony and high cluster number were judged as a "leukemic" growth pattern. All other cultures were considered "normal" growth of GM progenitors [25].

Statistical analysis

Computer-aided analysis was done with methods of descriptive statistics. The correlations between different parameters were assessed by linear regression test. Survival and AML development were plotted according to the Kaplan-Meier method and curves were compared by using log-rank test. P-values<0.05 were considered statistically significant.

Results

Among 39 patients enrolled in this study, there were 24 males and 15 females, median age 62 years (range 32-84). The median age of controls was 57 years. According to FAB classification [16], 13 patients had low risk MDS (RA/RARS), whilst 26 patients had advanced disease (17 patients with RAEB/RAEB-t and 9 patients with chronic myelomonocytic leukemia [CMML]).

Analysis of antigen expression

Expression of early and lineage unspecified antigens (CD34, CD38, HLA-DR), "early" (CD13, CD33) and "late" myeloid cell-associated antigens (CD11b, CD15), monocyte (CD14), megakaryocyte (CD41) and erythroid marker (Glycophorin A) was analyzed and compared to the healthy control group (Table 1). A distinct finding was a significantly higher proportion of cells expressing CD13, CD33, CD14, CD15 and CD38 antigens on bone marrow myeloid compartment in MDS patients, compared to findings in the control group. Although the average expression of HLA-DR, GpA, CD41 and CD34 antigens was higher than in the control group, this difference did not reach statistical significance, mainly due to the considerable individual variability among patients.

CD13 antigen was expressed in higher proportion of bone marrow myeloid cells with high frequency in the whole MDS group, regardless of the disease stage (i.e. in 12/13 low risk patients and 23/26 patients with advanced disease). CD33 antigen was expressed in higher proportion of bone marrow myeloid cells in 10/13 low risk patients and 17/26 patients with advanced disease. Although elevated proportion of HLA-DR^+ and $CD34^+$ bone marrow myeloid cells was present with lower frequency in low risk patients (3/13 patients and 2/13 patients, respectively), compared to patients with advanced disease (13/26 patients and 11/26 patients, respectively), these differences were not significant (p=0.107 and p=0.09, respectively). Uniquely consistent correlation was found in case of CD14 antigen, which was found in higher proportion of bone marrow myeloid cells in all 9/9 (100%) CMML cases, compared to 2/13 (15%) RA(RS) patients and 7/17 (41%) RAEB(T) patients (p=0.04). The proportion of $CD11b^+$ cells was lower in 4/13 (31%) low risk patients, compared to 11/26 (42%) patients with advanced disease. GpA and CD41 antigen were occasionally expressed in higher proportion of bone marrow myeloid cells, regardless of the FAB subtype (4/13 low risk patients and 7/26 patients with advanced disease; and 9/13 low risk patients and 5/26 patients with advanced disease, respectively). All these differences were not significant.

Relationship of antigen expressions with complete blood counts and marrow blast count

Analysis of linear correlation matrices showed that CD13 antigen expression was in positive linear correlation with HLA-DR and CD14 antigen expression (r=0.497, p=0.0017 and r=0.623, p=0.00006, respectively), the percentage of bone marrow blasts (r=0.393, p=0.016) and white blood cell counts (WBC) (r=0.395, p=0.015). In contrast, similar correlations were not found in case of CD33 antigen expression. CD14 antigen expression was in positive linear correlation with CD13 antigen expression (see above), HLA-DR antigen expression (r=0.444, p=0.0067), WBC (r=0.806, p=0.000001) and monocyte count in peripheral blood (r=0.72, p=0.000002). Expression of CD11b and CD15 antigens was inversely correlated with the expression

of HLA-DR antigen (r=-0.33, p=0.05 and r=-0.564, p=0.0033, respectively). GpA antigen expression was inversely correlated with hemoglobin concentration (r=-0.41, p=0.047), while CD41 antigen expression was in positive linear correlation with platelet count (r=0.616, p=0.0084). HLA-DR antigen expression was in positive linear correlation with CD13 and CD14 antigens (see above), the percentage of blast cells in bone marrow and peripheral blood (r=0.648, p=0.000008 and r=0.525, p=0.0006, respectively), and inversely correlated with CD11b and CD15 antigen expression (see above). CD34 antigen was expressed in a positive linear correlation with HLA-DR antigen expression (r=0.652, p=0.0004), and in a negative correlation with expression of CD11b and CD15 antigens (r=-0.48, p=0.014 and r=-0.564, p=0.0033, respectively). However, the proportion of $CD34^+$ BMMCs did not correlate with the proportion of morphologically recognized bone marrow blast cells. Yet, overexpression of CD34 antigen correlated with bone marrow dyshematopoiesis (Kruskal Wallis test, p=0.024).

Myeloid ratio

The myeloid ratio (HLA-DR/CD11b) was significantly higher in patients with MDS as compared with the control group (Table 2), particularly in patients with advanced disease (Figure 1). However, although the mean myeloid ratio in MDS patients was 2.5-fold higher than in the control group, this difference was not significant due to individual variability among patients. Elevated myeloid ratio (i.e. values >95% confidence range of the control group) had 15 out of 36 (42%) patients with MDS. Regarding FAB classification, patients with RA(RS) had significantly lower myeloid ratio in comparison to RAEB(T) patients (0.24±008 vs. 1.19±0.5, t=6.55, p<0.05) (Figure 1). The myeloid ratio was in positive linear correlation with the proportion of bone marrow blast cells (r=0.458, p=0.005).

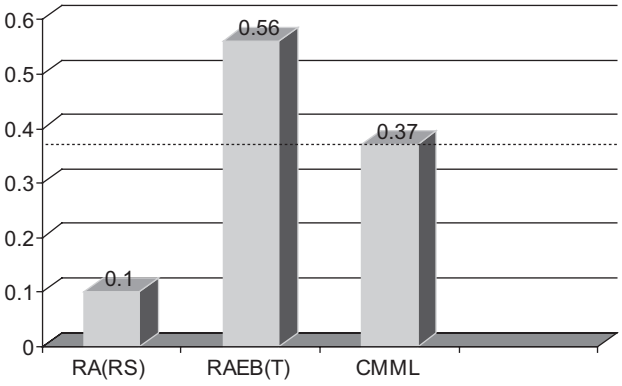

Figure 1. Median values of the myeloid ratio in different disease subtypes. Dashed line indicates value of 0.36 which is the upper limit of normal.

Relationship of immune phenotype with cytogenetics and colony forming cells

Although the average myeloid ratio and proportion of $CD34^+$ bone marrow cells in patients with abnormal karyotype and/or GM-progenitor growth were higher compared to patients without such abnormalities, these differences were not significant (Table 3).

Correlations to overall survival and evolution to overt leukemia

Patients with higher proportion of $CD34^+$ bone marrow cells (i.e. values >95% confidence range of the control group) and patients with proportion of $CD34^+$ bone marrow cells within normal range were compared regarding overall survival and risk for leukemic transformation. The median survival of patients with higher proportion of $CD34^+$ bone marrow cells was significantly shorter compared to patients with proportion of $CD34^+$ bone marrow cells within normal range (7 vs. 31 months, p=0.0375) (Figure 2). Patients with higher proportion of $CD34^+$ bone marrow cells had significantly higher leukemic transformation (LT) rate, as well (median time to LT 24 vs. >25.0 months, p=0.046). The median survival of patients with elevated myeloid ratio was significantly shorter compared to patients with myeloid ratio within normal range (7 vs. 33 months, p=0.013) (Figure 3). Patients with elevated myeloid ratio had significantly higher LT rate, as well (median time to LT 14 months vs. not reached, p=0.041).

Table 3. Relationship of immune phenotype with karyotype and growth pattern of GM progenitors in patients with MDS

Immune phenotype	*Karyotype**		*Growth of GM progenitors***	
	Normal	*Abnormal*	*Normal*	*Abnormal*
Myeloid ratio				
x ± SEM	0.42±0.1	1.06±0.6	0.36±0.11	0.5±0.2
median	0.21	0.35	0.17	0.28
(range)	(0.03-1.5)	(0.08-7)	(0.05-1.27)	(0.03-1.5)
CD34 expression				
x ± SEM	8.1±2.5	6.9±1.8	5.8±2.3	9.3±5.4
median	2.0	5.0	4.0	4.5
(range)	(0-36)	(4-19)	(0-19)	(1-36)

*normal vs. abnormal myeloid ratio and CD34 expression: p=0.8 and p=0.37, respectively, **normal vs. abnormal myeloid ratio and CD34 expression: p=0.7 and p=0.98, respectively. SEM: standard error of the mean

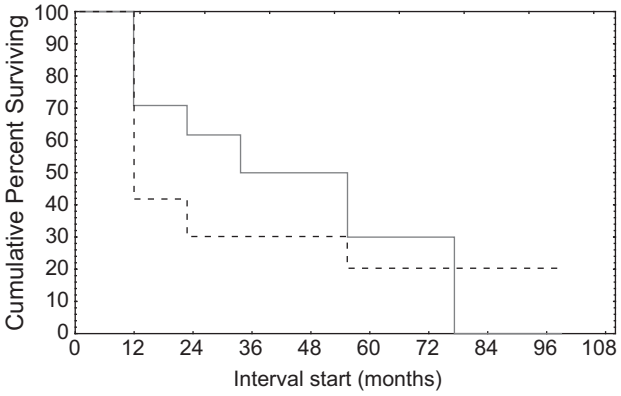

Figure 2. Kaplan-Meier plots predicting survival: patients with CD34 overexpression (solid line) compared to cases with CD34 expression within 95% confidence range of the control group (dashed line). The difference is significant (p=0.0375).

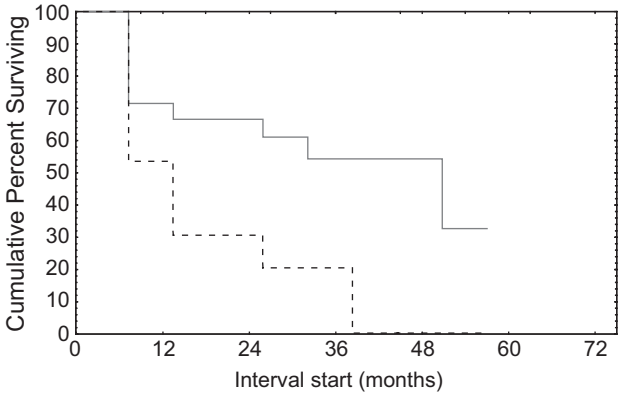

Figure 3. Kaplan-Meier plots predicting survival: patients with elevated myeloid ratio (solid line) compared to cases with myeloid ratio within 95% confidence range of the control group (dashed line). The difference is significant (p=0.013).

Discussion

Basic morphology in combination with cytogenetic findings give crucial information to render a diagnosis in most of MDS cases [26]. Multiparameter flow cytometric evaluation of bone marrow population, as a first line diagnostic procedure for MDS, is not recommended by current diagnostic proposals, whereas some of the reasons are extensive and expensive panels of monoclonal antibodies for detailed immunophenotypic analysis [1,27]. However, in cases in which morphology and cytogenetics are indeterminate and repeated analyses are not informative, flow cytometric immunophenotyping, as an accurate and highly sensitive method for detection of quantitative and qualitative abnormalities of hematopoietic cells, can help establishing the diagnosis of MDS [2,3]. Nevertheless, the purpose of this study was to discuss the potential contribution of flow cytometric evaluation of bone marrow myeloid compartment to better understand the pathobiology of MDS.

The results of our immunophenotypic study showed that in the BMMNCs of the majority of MDS patients there is maturational left shift, disclosed as an elevated proportion of myeloid cells expressing early differentiation and myelo-monocytic antigens (HLA-DR, CD38, CD13, CD33, CD15), compared to the expression pattern of healthy control BMMNCs. In contrast to individually variable CD33 antigen expression, CD13 antigen was the most consistently expressed myeloid antigen in the myeloid compartment cells in MDS patients, which is in accordance with other reported studies [6,8,10,28]. Moreover, the proportion of CD13+ myeloid cells correlated significantly with some parameters of biological aggressiveness of the malignant clone (proportion of HLA-DR$^+$ myeloid cells as well as proportion of bone marrow blasts). Although all of CMML cases had significantly higher proportion of monocytic CD14$^+$ cells inside the myeloid compartment, the population of CD14$^+$ cells was also elevated in a proportion of patients with other MDS subtypes, particularly with RAEB(T) subtypes. These findings, together with established correlations between CD14 and CD13, as well as CD14 and HLA-DR antigen expression, indicate involvement of the myelo-monocytic lineage in advanced disease.

Elevated proportion of bone marrow myeloid cells with expression of erythroid (GpA) or megakaryocyte (CD41) antigens was found in a small number of patients. However, the proportion of GpA$^+$ cells was in positive correlation with the proportion of CD34$^+$ cells and in negative correlation with hemoglobin concentration, suggesting accumulation of "immature" erythroid cells, maturational "block" and ineffectiveness of erythropoiesis in MDS. Namely, in our previous study in which we analyzed the growth pattern of hematopoietic progenitors in MDS [29], we showed significant correlation between the number of BFU-E and hemoglobin concentration and presumed that anemia in MDS is generated mainly due to deficit of the population of BFU-E cells. We also showed significant correlation between the number of CFU-MK and platelet count [29], which, together with the established positive linear correlation between the proportion of CD41$^+$ cells and the platelet count in this study, may suggest that the effectiveness of megakaryocytopoiesis in MDS depends not only upon the maturation capacity of progenitor cells, but also upon the size of precursor "pool".

We found that increased proportion of CD34$^+$ BMMNCs is associated with poor outcome, as already reported [10-12]. In contrast to the results of Gyotat et al. [10], we did not find positive correlation between the proportion of CD34$^+$ BMMNCs and bone marrow blast

count. However, sometimes relevant discordance rate between morphology, flow cytometry and immunohistochemistry methods exists regarding the estimation of bone marrow blast counts in MDS [30]. Moreover, processing of the marrow sample for flow cytometry analysis additionally complicates comparison between the visual blast count and the CD34 value [31]. The frequency of patients with elevated proportion of HLA-DR+ cells in the bone marrow myeloid compartment was higher in our patients with advanced disease, in accordance with other reports [8,20]. This fact substantially contributed to the prognostic significance of the "myeloid ratio" (HLA-DR/CD11b), since CD11b antigen was uniformly expressed among patients, regardless of the disease stage. However, immunophenotypic findings ("elevated" myeloid ratio and higher proportion of $CD34^+$ cells) were unrelated to the presence of chromosomal aberrations and to abnormal CFU-GM colony growth. This finding additionally contributes to the value of immunophenotype in predicting the outcome in patients in whom the karyotype is normal or insufficient number of mitoses is obtained [12].

Conclusion

Detection of immunophenotypic abnormalities of bone marrow myeloid cells, exhibited as abnormal expression of cell surface antigens, gives important insight to our understanding of MDS biology, disclosing important relations between immunophenotypic abnormalities and clinical features, and clearly contribute to accurate prognosis. Particular importance had the presence of increased proportion of $CD34^+$ BMMNCs and elevated myeloid ratio. However, further immunophenotypic studies with particular attempt to elucidate the complex and combined patterns of antigen expression with more powerful prognostic significance are necessary and will hopefully improve the clinical approach to these intractable disorders.

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