The Role of Flow Cytometry in Myelodysplastic Syndromes

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Flow cytometry has played a major role in the clinical laboratory in counting CD4 cells for monitoring HIV progression and CD34 cells for hematopoietic stem cell transplantation (SCT). It has also aided in classifying leukemias and lymphomas and in detecting low levels of residual disease after therapy. However, the flow cytometric assessment of a major monomorphous cell population (as occurs in phenotyping leukemias) or the detection of blasts when the phenotype is known (in residual disease detection patterning the diagnostic specimen), is different from that which must be used in diagnosing and determining the prognosis of myelodysplastic syndromes (MDS).

When used in a diagnostic role for unexplained cytopenias in suspected MDS, the manner in which the technology is applied changes to a search for an abnormality causing or related to the inadequate production of hematopoietic cells. Flow cytometric data for MDS must be used in the same way as a morphologic review of an aspirate smear, comparing the features of normal (non-neoplastic) bone marrow elements to their neoplastic counterparts. Each lineage must be examined to determine the frequency of cells, degree of maturation, and suspected abnormalities observed relative to previous knowledge of cytologic appearance. A flow cytometric approach requires an intimate knowledge of which antigens are present on each lineage at each maturational stage and how their expression is related to other cellular antigenic markers. With proper selection of reagents and a careful multidimensional analysis, identifying every cell in a marrow aspirate specimen, classifying it to a lineage and a maturational stage within that lineage, and assessing whether or not it displays a normal or an abnormal phenotype is possible.

The cell surface antigens detected by monoclonal antibodies are translated gene products, predominantly glycoproteins, that are highly regulated during maturation...
from hematopoietic stem cells to fully functional mature cells seen in peripheral blood. This regulation of gene product expression is characterized not only by the precise sequential appearance and disappearance of cellular antigens but also by the quantitative amounts of these products detected on the cell surface or in the cytoplasm. In addition, differential gene splicing is required for expression of different molecular isoforms of some genes.

Cells regulate the amounts of gene products by complex intra- and intercellular mechanisms, resulting in the quantitative expression of antigens precisely controlled in normal hematopoiesis, such as are described for the regulation of CD13 by inhibitory c-Myb and c-Maf complexes.  

Another feature of the steady-state development of normal hematopoietic cells is that the multiple changes that mark progression of maturation occur simultaneously. This is best demonstrated in the development of the lymphoid cells (both B and T cells) but is also seen among the myeloid cells. During the maturation of the cell, some gene products are expressed at specific time points while others are suppressed. At these same time points, other gene products can be up- or down-regulated. Observations of this flow cytometric phenomenon led to 2 important conclusions: 1) hematopoiesis can be described as a series of steps, with progression from one stage to the next characterized by multiple intensity changes in gene products or antigenic expression; and 2) maturation of hematopoietic cells requires the simultaneous coordination of expression of multiple gene products. This coordination of expression of gene products requires the synchronized control or regulation of diverse processes—on/off/up/down/differential splicing—occurring at precisely the same time in development.

The stepwise changes have the characteristics of a molecular switch, coordinating the changes to allow developing cells to progress to the next stage. Moreover, individual genes can be regulated more than once in the development of cells of a specific lineage. For example, CD13 is present at an intermediate level on myeloblasts, and is upregulated in the development of neutrophils then shut off just before the promyelocyte stage, only to be turned on again at the metamyelocyte stage and later expressed at high levels on the mature neutrophil. These stages on normal hematopoietic cells, defined by the intensity of expression of multiple cell surface antigens, are invariant with age and remain intact in stressed or regenerating bone marrow after chemotherapy or even bone marrow transplantation.

**Phenotypic Changes in Neoplastic Bone Marrow**

A complete understanding of the antigen expression on normal maturing lymphoid and myeloid cells made it possible to compare the phenotypes of leukemic cells to understand how the developmental patterns were disrupted. The first studies compared the phenotype of 120 pediatric acute lymphoblastic leukemia (ALL) cases to examples of normal development. The researchers found that none of the specimens exactly matched the relationships in antigen expression patterns that were seen in normal instances. Each pattern, although similar in overall phenotype, was unique in the intensity and combinations of antigens expressed.

This finding was extended to acute myeloid leukemia (AML) with a detailed analysis involving 100 patients. As with ALL, the antigen patterns expressed on the blast cells in AML did not fit into the normal developmental sequence, and each leukemic phenotype was unique. The blast populations were often heterogeneous in antigen expression, with abnormalities identified in both the neutrophil and monocyte lineages.

Multiple studies have shown that the phenotypes of bone marrow cells in patients with MDS exhibit differences from the patterns identified in normal bone marrow, not only among the blast cells, but also on the maturing myeloid cells. These abnormalities are seen on both myeloid and monocytic lineages and, as with the patterns in AML, the constellation of antigens expressed or misexpressed are heterogeneous and unique to each patient. Therefore, the flow cytometric assessment of MDS reveals the loss of the precise regulation and co-regulation of myeloid antigens during development of both the maturing myeloid cells and of the immature blasts. The variability in published reports on flow cytometry in studying MDS is caused, in part, by the uniqueness of each patient and the parameters used to distinguish between “normal” presentation of antigens and those seen on each unique patient. Because of the extensive variability from patient to patient as well as the changes that occur over time for an individual patient, single specific antigenic changes are not sufficient to fully capture the extent
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of regulation loss. The abnormalities seen are not just in the expression of single antigens but affect the relationships between multiple antigens. Panels of monoclonal antibodies must be integrated so researchers can focus on the steps of myeloid and monocyte development in which multiple gene products are co-regulated and thus note the loss of coordination of regulation of gene products at multiple steps during development. This analysis must distinguish between a shift-to-the-left in stressed marrows and a true uncoupling of the co-regulation of gene products in neoplastic processes that may occur at any of the developmental steps. The details of data analysis are beyond the scope of this manuscript; however, a summary of the common types of abnormalities seen in patients with myelodysplasia are listed in Table 1.

These abnormalities are similar to those detected in AML and include inappropriate lineage expression (lymphoid antigens on myeloid cells), over- or under-expression of an antigen, asynchronous expression where antigens normally identified on immature cells appear on mature cells, and complete absence of antigen expression (possibly because of complete loss of the gene, regulators, or overinhibition). Other abnormalities can be seen in physical changes of cell size (detected by forward light scatter) and granularity (identified by right angle light scatter or side scatter, where the included granules in myeloid cells act like tiny mirrors reflecting light into the detector). Phenotypic abnormalities on the myeloblasts have been studied on total CD34 positive cells, immature or maturing CD34 positive cells, or the entire maturing myeloblast population independent of CD34 expression.

Flow cytometry analysis (focusing on normal/abnormal blasts, myeloid and monocyte development) to detect phenotypic abnormalities (phenotypic dysplasia) can be complementary to morphologic analysis of bone marrow. Dyspoietic features in the erythroid and megakaryocytic lineages are often prominent by morphology, whereas the assessment of abnormal myeloblasts, maturing myeloid cells, and especially monocytes, is more difficult by morphology. Together the 2 technologies can provide a better total picture of the bone marrow in MDS patients.

Patients classified as having refractory anemia with no morphologic dyspoiesis identified in the myeloid cells often have phenotypic abnormalities on either the monocytes or developing myeloid cells, changing the classification to refractory cytopenia with multi-lineage dysplasia. Technical considerations limit the analysis of erythroid and megakaryocytic cells by flow cytometry. Megakaryocytes are too

| Table 1 Commonly Observed Flow Cytometric Abnormalities in Myelodysplastic Marrows* |
|----------------------------------------|----------------------------------|----------------------------------|
| **Myeloblast Abnormalities** | **Maturing Myeloid Abnormalities** | **Monocyte Abnormalities** |
| Absence/decreased HLA-DR, CD45, CD34, CD13, CD33, CD117 | Abnormal granularity | Abnormal granularity |
| Overexpression of CD34, CD13, CD33, CD117 | Abnormal decrease in CD45 expression | Abnormal CD11b/HLA-DR relationship |
| Expression of CD56, CD7, CD5, CD11b | Presence of HLA-DR | Loss of CD13 |
| Expression of other lymphoid antigens | Convex or abnormal relationship between CD13 and CD16 | Lack of CD14 (must distinguish from PNH) |
| | Asynchronous shift to the left | Lack of CD33 expression (may be a normal variant) |
| | Absence of CD16 (must distinguish from PNH) | Expression of CD56 (may be non-specific finding) |
| | Absence or decreased CD33 (may be a normal variant) | |
| | Absence or decreased CD11b, CD10, CD64, CD65 | |
| | Expression of CD56 (may be non-specific finding) | |

Abbreviations: HLA-DR, human leukocyte antigen-D-related; PNH, paroxysmal nocturnal hemoglobinuria.

*Decreased or increased intensities of antigen expression require 0.5 log difference from normal in multidimensional space or comparison to a reference range for each antigen on normal specimens.
infrequent to be analyzed by flow without extensive enrichment. Analysis of erythroid cells is hampered by the requirement to remove mature red blood cells from analysis, which variably affect earlier forms as well (erythrocyte lysis or Ficoll). In addition, erythroid development is relatively simple with respect to steps of maturation (2 distinct stages); most antigens are lost during maturation, including CD45, HLA-A/B/C, HLA-DR, and CD117. With regards to MDS, cytoplasmic antigens such as fetal hemoglobin or ferritin may be more useful in detecting dyserythropoiesis than focusing on the cell surface.

Identifying, Enumerating, and Classifying Blasts

Flow cytometry has a distinct advantage over morphology for blast cell enumeration because of the ability to count many more cells (≥ 10,000 as opposed to 300–500). Another advantage is a definable set of cellular characteristics that can be used to identify cells instead of the possibly subjective morphologic classification of a blast. Because several different combinations of reagents can be used to discriminate the myeloblasts, researchers have recommended that a phenotypic blast count should be obtained using multiple combinations of markers, comparing the results for consistency and as an internal quality check of the data. When applied to MDS, the identification and enumeration of blasts must incorporate the multiple reagent combination approach because the process of neoplastic transformation may involve the loss of one or more of the antigens used to classify the cells as immature.

The most commonly used antigen to identify blasts in bone marrow, peripheral blood, or other tissues is CD34, an antigen expressed on progenitor cells but not on mature cells. CD34 is expressed on the most immature of the myeloid blasts and is lost early in myeloid development. Therefore, CD34-positive myeloblasts may not represent the identical population counted by morphologists. Although CD34 is expressed on normal blasts, its expression can be lost on immature cells in some MDS cases or, in some instances, it can be found on more mature myeloid or monocytic cells.

Other features of CD34 make it less desirable as the sole means of defining blasts. Immature B lymphoblasts, stage 1, express high levels of CD34 and must be distinguished from immature myeloid cells counted by the morphologists. Therefore, lymphoblasts must be excluded from the analysis. Likewise, platelets can express dim CD34 and can form complexes with leukocytes, causing a spurious count of the immature cells, especially in cases of myeloproliferative disorders. Basophils also express low levels of CD34 and are often erroneously included in CD34 counts.

Another combination, CD11b/HLA-DR/CD45, may be more useful for identifying granular blasts than CD34, but this combination is subject to some of the same problems as CD34 in that a correction must be made for immature B lymphoblasts (as well as mature B lymphoid cells, activated T cells, and NK cells). HLA-DR may be absent from blasts in some MDS patients and CD11b is sometimes expressed on blasts that are maturing to monocytes. Another antigen, CD117, is expressed only on myeloblasts, not lymphoblasts, but it is lost at a slower rate than the other antigens and can still be detected on early myeloid cells just acquiring primary granules and at high levels on mast cells. Therefore, the use of 3 or more combinations provides a redundant system with internal controls for the loss or inappropriate expression of any one of the markers.

A major difference exists between the calculation of blast counts used for flow cytometry and morphology. Although nucleic acid stains can be used to identify nucleated cells in the presence of intact erythrocytes by flow cytometry, the numerous mature erythrocytes distort the light scatter signals. Therefore, erythrocytes must be removed from the analysis of bone marrow specimens, usually by lysis with NH4Cl or density separation (Ficoll). These procedures have different effects on nucleated erythrocyte precursors. Ficoll retains the erythroid cells through the reticulocyte stage whereas NH4Cl lysis depends on the activity of carbonic anhydrase which appears midway through erythroid development. Ficoll preparation also eliminates the mature myeloid cells, potentially biasing the analysis of the myeloid cells. Therefore, the preferred preparation technique is lysis of the erythroid cells necessitating flow cytometric blast counts to be expressed per non-erythroid cell, that is, per CD45 positive cell. This approach is identical to that used for blast counts in the FAB M6 category when erythroid precursors are greater than 50% of all cells. Flow cytometric analysis makes this correction on all specimens rather than just for the M6 category.
An additional complication for using flow cytometric blast counts arises because the bone marrow aspirate has a variable amount of blood contamination. A method for the assessment of blood contamination in marrow aspirates was recently proposed.17

In addition to blast counts, a precise phenotypic analysis permits the detection of abnormal blasts not only for higher blast counts but also for blasts less than 5%. The variability of antigen expression on blasts in MDS is similar to that seen for AML; however, the proportion of blasts is proportionally decreased. At diagnosis, such an analysis falls under the category of minimal disease detection without a prior diagnostic specimen, requiring an intimate understanding of the expression of a variety of antigens to identify subtle abnormalities based on “difference from normal.” A study of low-risk MDS comparing the phenotype of only the CD34 positive cells showed that more than half of patients with morphologic blasts less than 5% showed phenotypic abnormalities.17

A recent detailed analysis of CD34-positive cells in bone marrow that distinguish immature from more mature myeloblasts showed significant intensity differences for a variety of antigens in all MDS cases compared with non-neoplastic specimens.24 Similar results were obtained in a separate study in which abnormalities on the blast population (defined in multidimensional space) were also found in most cases, independent of the maturing myeloid cells.25 The detection of abnormalities in the myeloblast population, even at low levels, appears to be a clear sign of some transformation or genetic dysregulation and facilitates the identification of patients with MDS.17,19,24

Understanding the Uniqueness of Each Patient

The heterogeneity of antigen expression in MDS has placed a significant conceptual obstacle in grouping patients based on flow cytometric findings. Because no single antigenic abnormalities have been correlated to an accurate diagnosis or prognosis, simply counting the number of abnormalities for each patient provides a means of measuring the distance the marrow cells have evolved from normal.19,21,25 The assumption is that a patient with multiple phenotypic abnormalities—reflecting more extensive loss of gene product regulation—shows more disease progression than a patient with fewer abnormalities. Combining phenotypic abnormalities on both the blasts and maturing myeloid cells may have a useful role in diagnosing patients with myelodysplasia.13,19,21,25

The extent of dysregulation identified by phenotypic abnormalities may also be useful for prognosis. One scoring system was devised based on comparing the patterns of antigen expression in MDS patients to those in stressed marrows assessed early after SCT or in patients with previously treated Hodgkin lymphoma, aplastic anemia, or after chemotherapy for non-hematologic diseases.21 Myeloid development was separated into 3 separate compartments: myeloblasts, maturing monocytes, and maturing neutrophils. The myeloblasts were scored either as normal (0) or abnormal less than 5% (1), with additional points added for blast counts greater than 5%.

Abnormal features on maturing monocytes were scored separately from the abnormalities on maturing myeloid cells. The goal was to assess each of the steps of maturation for these lineages to detect the dysregulation occurring during the entire process of development of monocytes and neutrophils. A single antigenic feature was assigned a score of 1, reflecting the loss of regulation of that 1 feature. In the initial study of patients with MDS coming for SCT, the specimen just before transplantation was scored in a blinded fashion, without knowledge of other clinical data or outcomes of the patients. The flow score was inversely correlated to absolute neutrophil count and was directly related to the International Prognostic Scoring System (IPSS).23 This indicated that the number of abnormalities was related to decreased neutrophil production and to other well-documented clinical findings (i.e., karyotype, number of cytopenias, and blast count).

The scoring system was useful in distinguishing normal or stressed marrow from marrow in MDS. Using a flow score of 3, the assessment was 100% specific but only 51% sensitive. Lowering the flow score to 2 reduced the specificity to 91% but improved the sensitivity to 73%. The flow score was also highly correlated to relapse and survival rates after allogeneic SCT. Moreover, patients with an intermediate-1 category by IPSS could be further stratified by flow score, with significantly worse prognosis seen with higher flow scores. This study suggests that the amount of dysregulation in gene product expression (number of antigenic abnormalities) correlates with more severe disease and that the extent of dysregulation may be an entirely
A significant number of patients with a morphologic classification of refractory anemia in the World Health Organization (WHO) classification were identified as having aberrancies on the maturing myeloid or monocytic cells. Therefore, they were more appropriately classified as refractory cytopenias with multilineage dysplasia. This result suggests that flow cytometric analysis may be more sensitive to dysplastic features on the myeloid or monocytic cells compared with morphology. A significant correlation was seen between the flow cytometric score and WHO classification. The flow cytometric score was not related to specific cytogenetic risk groups but was related to the overall IPSS and to the WHO classification-based prognostic scoring system. The flow cytometric score correlated with transfusion dependency and disease progression to refractory anemia with excess blasts-1 within 18 months. The expression of lymphoid antigens on the myeloblasts was also related to transfusion dependency. These different studies suggest that accumulation of phenotypic abnormalities is a distinct, separate parameter for assessing patients with MDS, which may further subdivide a heterogeneous group of patients with respect to clinical outcomes.

### Moving Forward

For flow cytometric data to be routinely used in the treatment of MDS patients, consensus must be obtained for counting myeloblasts using standard reagent panels and protocols that also extend to the enumeration of abnormal blasts. Defining reproducible criteria is necessary to discriminate normal from abnormal myeloblasts and aid in diagnosing early-stage MDS. The assessment of antigen expression on maturing myeloid cells must also be standardized, defining an “abnormal feature” that reflects gene dysregulation rather than marrow stress. Then, large prospective clinical studies must be completed to better define weighting factors to be used for each abnormality and that can be used to generate a flow score that is easy to obtain, highly reproducible, consistent from laboratory to laboratory, and useful in both diagnosis of and prognosis for MDS patients.

### References

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