Diagnostic flow cytometry for low-grade myelodysplastic syndromes

Kiyoyuki Ogata*
Division of Hematology, Department of Medicine, Nippon Medical School, Tokyo, Japan

*Correspondence to: Dr. Kiyoyuki Ogata, Division of Hematology, Department of Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan. E-mail: ogata@nms.ac.jp

Abstract

It has long been considered that flow cytometry (FCM) has little role in clinical practice in the diagnosis of myelodysplastic syndromes (MDS). However, recent advances in the analytical method and knowledge of MDS FCM are changing this stereotype. This paper reviews the concept and current status of FCM in the diagnosis of low-grade MDS. The diagnosis of low-grade MDS in the absence of ringed sideroblasts and chromosomal aberration is not always straightforward, and a report from a recent international working conference has proposed FCM as an adjunctive diagnostic test for such cases. Currently, only a limited number of laboratories are applying FCM to the diagnosis of MDS. Furthermore, standard analytical methods in FCM for MDS have not been established, and no single FCM parameter is sufficiently sensitive and specific to make the diagnosis of MDS. To establish MDS FCM as a widely accepted, dependable diagnostic tool, prospective studies should increase flow parameters that can be analysed reproducibly and determine their sensitivity and specificity, either alone or in combination. CD34+ cell-related parameters that are applicable for diagnosing low-grade MDS in many laboratories are introduced here. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: myelodysplastic syndromes; flow cytometry; diagnosis; CD34

Introduction

Myelodysplastic syndromes (MDS), which were originally defined by the French–British–American classification [1] and later refined in the World Health Organization classification [2], are malignant disorders of haematopoietic cells. The bone marrow (BM) in MDS is composed of clonal myeloid cells showing various degrees of differentiation in each case. MDS usually exhibit cytopenia, mainly due to the early death of partially or fully differentiated myeloid cells and insufficient differentiation capacity of the progenitors to transform into mature blood cells [3,4]. The prognosis of MDS is poor [5–7]. Manifestations caused by cytopenia and transformation to acute myeloid leukaemia (AML) due to further loss of the ability of clonal cells to differentiate are the major causes of death in MDS [3,4]. MDS appear to be the most common myeloid malignancy and their incidence increases steeply with age [8,9]. Therefore, MDS are becoming more important in health care in developed countries.

A report from an international working conference in Vienna in 2006, in which experts in MDS participated, proposed refined definitions and standards for the diagnosis and treatment of MDS [10]. In that proposal, flow cytometry (FCM) was considered to be an adjunctive diagnostic test. Meanwhile, it was the consensus of that conference that standard analytical methods in FCM for the diagnosis of MDS remained to be established [10,11].

Issues in MDS diagnosis

The diagnosis of MDS is based on a combination of clinical history, the morphologic features of the peripheral blood (PB) and BM (e.g. percentages of blasts and dysplastic features of cells), cytogenetic data and ruling out other diseases [1,10]. The diagnosis of MDS is straightforward if clearly objective abnormalities, that is, increase in blasts and/or ringed sideroblasts and/or presence of chromosomal aberration as evidence of clonal myelopoesis, are detected. In other words, a diagnostic challenge exists in low-grade MDS without ringed sideroblasts (LGw/oRS) with normal karyotype. For example, although the diagnosis of LGw/oRS with normal karyotype largely relies on the presence of dysplasia, how do we distinguish clearly between dysplastic myelopoiesis and normal myelopoiesis? Obviously, experienced examiners (haematologists/haematopathologists) are required to make this distinction. However, even if experienced examiners succeed in correctly judging that 10% of erythroid cells are dysplastic in patients, which is a criterion defining dyserythropoiesis [10], it is uncertain whether patients in whom 9% of erythroid cells are dysplastic do not have MDS. Moreover, the dysplastic features of myeloid cells do not in themselves establish a diagnosis. There are conditions other than MDS which can induce dysplastic myeloid cells (e.g. deficiencies of vitamin B12 and folate, viral infections and exposure to antibiotics, chemotherapy agents, alcohol, benzene or lead), and thus such...
Flow cytometry in MDS: background

FCM has been a routine clinical tool for diagnosing acute leukaemia and non-Hodgkin’s lymphoma, although its diagnostic value has not been established in MDS. What should MDS FCM be based on to become universally applicable? First, the parameters used in MDS FCM should have sufficient specificity and sensitivity. Second, the data of these parameters should be reproducible even if different flow operators, and hopefully even if different laboratories, analyse them. Finally, these parameters should be easily understood by the majority of clinicians who use this diagnostic tool.

In the late 1980s to early 1990s, many laboratories examined the immunophenotype of MDS cells. Most investigated circulating lymphocytes (Ly) or BM mononuclear cells (BMMNCs) that were definitely MDS heterogeneous cell populations. For example, decreased CD3+ cells and CD8+ cells in circulating lymphocytes as well as decreased CD11b+ cells and increased CD13+, CD33+ and CD34+ cells in BMMNCs were reported to be a sign of disease progression and poor prognosis in MDS [15–23]. The reports of MDS immunophenotyping in that era were thoroughly reviewed by Elghetany [24].

More recently, Stetler-Stevenson et al. reported on the value of FCM in MDS diagnosis [25] They examined BM cells using three-colour FCM, applying a pattern-recognition approach. That is, based on knowledge of the normal patterns of haematopoietic cells in FCM, they identified abnormal patterns in MDS samples (such as hypogranulation of neutrophils detected by low side scatter (SSC) and the presence of CD64—granulocytes). They found that FCM could diagnose MDS patients as efficiently as the conventional morphologic approach. Using a similar pattern-recognition approach, other groups also detected MDS-associated abnormalities in FCM [26–29].

Issues in the pattern-recognition approach

There are problems in the pattern-recognition approach, however. First, this approach requires sufficient experience and knowledge of the normal (control) patterns of haematopoietic cells in FCM, which is the basis on which examiners identify abnormal MDS flow patterns. In other words, this approach is an FCM version of cytomorphology. This is one of the reasons why this approach makes it difficult to define reference ranges of the flow parameters used. The greatest problem clinicians face is in differentiating nonclonal, cytopenic patients from LGw/oRS patients. Therefore, the former patients should be the main control group in MDS FCM studies. A variety of conditions, including immunocytopenia, drug-induced anaemia and other secondary anaemia, fall into this control category. Obviously, there are many cases in which FCM patterns are intermediate between typical control patterns and typical MDS patterns. Therefore, to make the pattern-recognition approach widely accepted and applicable in many laboratories, sufficient data from control individuals should be examined and appropriate reference ranges established [30].

Quantitative FCM for diagnosing MDS

Overview

Some laboratories have been working to quantify FCM parameters for MDS diagnosis. Using PB samples, Cherian et al. quantified the SSC of granulocytes and CD11a, CD66, CD10 and CD116 expressions on granulocytes for 11 MDS patients and 20 control individuals including 16 nonclonal, cytopenic patients [31]. They found that the data from the two groups overlapped, although some MDS patients had data that clearly deviated from the data range of controls. Malcovati et al. quantified a variety of flow parameters of BM cells, such as CD34+ cells, CD56+ granulocytes and erythroblasts, for MDS patients, healthy donors, and cytopenic patients other than with MDS [32]. Similar to Cherian et al., they found that the data from those three groups differed but showed a substantial overlap. Therefore, they performed discriminant analyses, which developed functions incorporating data on seven parameters to identify MDS patients efficiently. Using the same strategy and examining different flow parameters, such as cytosolic ferritin subunits, Della Porta et al. from the same group developed a function to identify sideroblastic anaemia [33]. Our group has focused on blast immunophenotype and found that most MDS blasts are CD34+ and often exhibit a variety of aberrations as observed in blasts in acute leukaemia [34,35]. Quantifying a variety of parameters in the CD34+ cell compartment is useful in diagnosing LGw/oRS patients with or without karyotype abnormalities [36]. These findings are described in the subsequent sections. It should be remembered that at present no single parameter relating to the CD34+ cell compartment has sufficient specificity and sensitivity in diagnosing LGw/oRS patients, and therefore, the combined use of multiple parameters is necessary.

Quantification of CD34+ B-cell precursors

In the B-cell development process, CD34+CD10+CD19+/−CD20− cells (also called stage I haematogones) mature into CD34−CD10+/−CD19+CD20+ cells (stage II haematogones) and finally into CD34−CD10−CD19+CD20+ mature B cells. This development process was
well characterized by Loken et al. [37,38]. A substantial number of these cells are detectable in the normal BM, but their number decreases in BM containing neoplastic cells, such as in leukaemia and lymphoma [39]. Our group reported that this phenomenon also occurs in MDS: both stage I and II haematogones decrease in number in the BM of MDS patients [34], which was confirmed by Sternberg et al. [40]. The number of stage I haematogones is a useful parameter that can be quantified. I recommend that these cells be expressed as the percentage among all CD34+ cells but not in other types such as all nucleated cells, because the former are not affected by the dilution of BM samples with PB. Stage I haematogones can be identified by their unique position on the SSC versus CD45 plot (Figure 1) or by their CD34+CD10+CD19+/− phenotype. Relatively rare events that should be considered in their identification are that these cells show higher SSC than usual when they are duplicating and MDS myeloblasts from some patients show an aberrant CD34+CD10+ or CD34+CD19+ phenotype [41,42].

Data on stage I haematogones quantified using three methods (SSC and CD45 characteristics, CD34+CD10+ phenotype, or CD34+CD19+ phenotype) for 10 LGw/oRS patients and 10 nonclonal, cytopenic patients are

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**Figure 1.** Strategy to quantify three CD34+ cell-related parameters. Cells in a single test tube were stained with CD10-FITC (or CD19-FITC), CD34-PE and CD45-PerCP. All nucleated cells (cells in the R1 gate in (A)) were defined as all cells with size equal to or greater than that of lymphocytes. The cells in the R2 gate were displayed on the CD45 versus CD34 plot (B). The cells in the R3 gate in panel B were displayed as green dots on the CD45-versus-SSC plot (C). In panel C, CD34+ B-cell precursors (CD34+B) can be identified as the cell cluster with low SSC and separated from CD34+ myeloblasts (CD34+Mbl). The gate for the former cells can be confirmed based on CD10 positivity. Lymphocytes (Ly) can be gated on this plot as well. Then, the following three parameters with little interoperator variability can be analysed: (1) percentage of CD34+ myeloblasts among all nucleated cells; (2) percentage of CD34+B-cell precursors among all CD34+ cells and (3) Ly/Mbl CD45 ratio (MFI of lymphocyte CD45/MFI of CD34+ myeloblast CD45). As alternatives, ‘the percentage of CD34+B-cell precursors among all CD34+ cells’ can be analysed by determining the percentage of CD10+ cells (CD19+ cell percentage if cells were stained with CD19-FITC) among all CD34+ cells (D). The interoperator variability of these alternative methods has not been reported.
in this cell population. In spite of the above discrepancy, committed clearly to the B or myeloid lineage might exist lacked myeloid cell antigens and thus precursors not yet cells detected using the SSC and CD45 characteristics using the CD34+ these cells lacked CD19 expression [38,43], while the type detected fewer stage I haematogones because some of /C223 Copyright /C6 Compared with the method using the CD34+ phenotype, the method using the CD34+/C6 haematogones differ between these two patient groups summarized in Table 1. The percentages of stage I haematogones among all CD34+/C6 cells, which were determined using three methods for 10 nonclonal, cytopenic patients (controls) and 10 LGw/oRS patients. This study was approved by the Institutional Review Board of Nippon Medical School. LGw/oRS patients had chromosomal aberrations and/or clear dysplasia, with other diseases excluded by thorough examinations and follow-up data. Controls were diagnosed by thorough examinations and follow-up data including response to therapy.

Quantification of CD34+ myeloblasts and their CD45 expression

It should be noted that the gate for CD34+ myeloblasts must be set to exclude CD34+ B-cell precursors (Figure 1). When the number of CD34+ myeloblasts is expressed as the percentage of these cells among all nucleated cells, high percentages (greater than or equal to 2.4% in our previous report [36]) are very predictive of MDS rather than nonclonal cytopenia. Points to be considered include the value of this parameter becoming falsely low if BM samples are diluted with PB and the value possibly being high even in nonclonal BM samples if they are in the recovery phase from myelosuppression. Also, the step of lysing erythrocytes in cell samples for FCM may falsely increase or decrease the data on ‘all nucleated cells’. Unlysed erythrocytes may remain, for example, due to reticulocytes, which resist lysing [45], or the poor quality of the lysing solution; ammonium chloride solution, which is commonly used to lyse erythrocytes, becomes ineffective over time [46]. In contrast, fragile erythroblasts (e.g. apoptotic erythroblasts) may paradoxically be lysed by exposure to the solution used to lyse erythrocytes.

Multiple groups reported that the CD45 expression of myeloblasts is decreased in some MDS patients [26,47]. Based on those observations, our group quantified this parameter by calculating the Ly/Mbl CD45 ratio (mean fluorescence intensity (MFI) of lymphocytes stained with anti-CD45 antibody/MFI of CD34+ myeloblasts stained with anti-CD45 antibody). The data on the Ly/Mbl CD45 ratio are not affected by the dilution of BM samples with PB. Importantly, by examining this quantifiable ratio, our group found that the CD45 expression of myeloblasts is not only decreased but also increased in some LGw/oRS patients [44].

### Table 1. Stage I haematogones quantified using three methods

<table>
<thead>
<tr>
<th>Group</th>
<th>Stage I haematogones quantified by</th>
<th>SSC versus CD45</th>
<th>CD34+CD10+</th>
<th>CD34+CD19+</th>
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<tr>
<td>Control 1</td>
<td></td>
<td>2.4</td>
<td>2.5</td>
<td>1.5</td>
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<tr>
<td>Control 2</td>
<td></td>
<td>8.1</td>
<td>7.1</td>
<td>3.7</td>
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<td>Control 3</td>
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<td>9.1</td>
<td>4.3</td>
<td>1.0</td>
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<tr>
<td>Control 4</td>
<td></td>
<td>14.8</td>
<td>8.0</td>
<td>3.4</td>
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<tr>
<td>Control 5</td>
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<td>22.1</td>
<td>18.7</td>
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<td>6.7</td>
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<td>10.7</td>
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<td>8.3</td>
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<td>Control 10</td>
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<td>33.9</td>
<td>17.0</td>
<td>4.1</td>
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<td>Mean ± SD</td>
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<td>20.5 ± 11.2</td>
<td>11 ± 6.0</td>
<td>4.4 ± 2.3</td>
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Data are percentages of stage I haematogones among all CD34+/C6 cells, which were determined using three methods for 10 nonclonal, cytopenic patients (controls) and 10 LGw/oRS patients. This study was approved by the Institutional Review Board of Nippon Medical School. LGw/oRS patients had chromosomal aberrations and/or clear dysplasia, with other diseases excluded by thorough examinations and follow-up data. Controls were diagnosed by thorough examinations and follow-up data including response to therapy.

### Points to be considered in analysing stage I haematogones using FCM

- Can be identified on the CD45 versus SSC plot or by CD34+/CD10+CD19+/− phenotype
- May decrease in the elderly
- Often decrease profoundly in the BM in not only MDS but also other neoplastic conditions
- Parameters not influenced by haemodilution (e.g. % of total CD34+ cells) are preferable

EDTA, ethylenediaminetetraacetic acid.

It is recommended that cell samples for FCM be heparinized and stored at 4–28 °C until use [49,50].

Patient age should be considered in data interpretation [39].

Chronic myelocytic leukaemia, lymphoma invading the BM, etc. [39,51].
These two CD34+ myeloblasts-related parameters are highly reproducible among different FCM operators, as are percentages of CD34+ B-cell precursors among all CD34+ cells [44]. These three parameters can be analysed in one test tube for three-colour FCM, in which BM cells are stained, for example, with anti-CD10-fluorescein isothiocyanate (FITC), anti-CD34-phycocerythrin (PE) and anti-CD45-peridinin chlorophyll protein (PerCP), and therefore can be conveniently examined in most laboratories. Combined use of these parameters can diagnose a certain percentage of patients with LGw/oRS with or without karyotype abnormality [44].

Quantification of other CD34+ myeloblast-related parameters

CD34+ myeloblasts in MDS overexpress or aberrantly express a variety of myeloid and lymphoid antigens. Our group quantified such antigens on CD34+ myeloblasts by calculating the relative mean florescence intensity (RMFI) (RMFI = MFI of each antigen staining/MFI of isotype-matched negative-control staining), which was also helpful in diagnosing patients with LGw/oRS with or without karyotype abnormality [36]. To make this approach widely applicable in many laboratories, data variation in the parameters examined by different FCM operators should be clarified and minimized.

Concluding remarks and future perspective

Recently, many laboratories have been working to develop MDS FCM and are still struggling to determine suitable flow parameters. Important points during this process include the following. First, the main group in whom clinicians are eager to use diagnostic FCM are LGw/oRS patients with normal karyotype. Therefore, flow parameters should be useful to identify them since flow characteristics probably differ between LGw/oRS and other MDS subtypes [34,47]. Second, the diagnosis of patients who participate in MDS FCM studies should be strictly considered and accurately defined. For example, the gold standard for LGw/oRS is patients who have chromosomal aberrations, who later transformed into AML, and/or who show clear dysplasia and have undergone thorough examinations and follow-up to exclude other diseases exhibiting dysplasia. The gold standard for controls is nonclonal, cytopenic patients whose diagnosis was confirmed by thorough examinations and follow-up data including response to therapy. Cytopenic patients in whom the diagnosis is unclear, including patients with idiopathic cytopenia with undetermined significance [10,48], should be separated from the above gold standards. To clarify the significance of FCM data in patients with uncertain diagnosis, long-term follow-up may be required. Third, using the above cohorts, the sensitivity, specificity and reproducibility of flow parameters should be clarified. I believe that determining and accumulating appropriate flow parameters will be achieved in the near future, which will ease, sharpen and deepen our approach to MDS.

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