Diagnostic Immunophenotype of Acute Promyelocytic Leukemia Before and Early During Therapy With All-trans Retinoic Acid

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ABSTRACT

Objectives: To study the immunophenotypic changes of acute promyelocytic leukemia (APL) in patients who recently received all-trans retinoic acid (ATRA) and to assess the diagnostic utility of flow cytometry in this setting.

Methods: Flow cytometry was performed on 29 newly diagnosed APLs and 93 other acute myeloid leukemias, including 25 HLA-DR– or CD34– cases. Clinical notes from referring institutions were reviewed to assess for recent ATRA administration.

Results: Recent ATRA therapy was documented in 17 (59%) of 29 patients with APL. The main features of untreated APL were preserved with ATRA therapy, including CD34– (83% vs 82%), HLA-DR– (83% vs 100%), and CD117+ (100% vs 77%). CD11b and CD11c were negative in all untreated APLs but positive in 76% and 88% of ATRA-treated APLs, respectively. Optimal diagnostic criteria for untreated APL (CD34– or HLA-DR– and CD11b– and CD11c–) showed 100% sensitivity and 98% specificity but were not useful after ATRA administration. The best interpretative approach to ATRA-treated APL (CD34– or HLA-DR–) showed 100% sensitivity but limited specificity (73%).

Conclusions: Information about recent ATRA administration is critical for adequate interpretation of the flow cytometric findings in patients with suspected APL.

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) with distinct morphologic, biologic, and clinical features, including the presence of abnormal promyelocytes with bilobed nuclei and frequent Auer rods. Most patients exhibit a diagnostic t(15;17)(q22;q21) balanced translocation, resulting in a fusion transcript joining the promyelocyte (PML) and retinoic acid receptor α (RARA) genes. APL represents a true emergency due to the high risk of developing life-threatening coagulopathy if not treated promptly. It is therefore widely recommended to emergently begin therapy with all-trans retinoic acid (ATRA) whenever the peripheral blood morphology and clinical features are suggestive of APL and before the diagnosis is confirmed by cytogenetic or molecular studies.
Multiparameter flow cytometry has an established role in the evaluation of newly diagnosed AMLs and is the method of choice for defining blast lineage.\(^5,6\) The applicability of flow cytometry to detect APL has been previously demonstrated,\(^7-12\) providing a rapid diagnostic tool when morphologic evaluation by a trained professional is not readily available. By flow cytometry, APL typically displays most of the following immunophenotypic features, including high side scatter; positivity for CD13, CD33, and CD117; and absent expression of CD34, HLA-DR, CD10, CD11a, CD11b, CD11c, and CD18. Some of these features might be absent in APL or present in other types of AML, thus requiring detection of the \(PML-RARA\) translocation by cytogenetic and/or molecular studies for final diagnosis.

Specialized cancer facilities often admit patients with a presumptive diagnosis of APL who were administered ATRA at the referring institution to prevent severe bleeding complications. Flow cytometry is widely considered a mandatory component in the initial workup on newly diagnosed acute leukemias to establish cell lineage and define the indicated chemotherapeutic regimen. Yet, the expected immunophenotype of APL early during ATRA therapy and the utility of flow cytometry in this setting have not been systematically studied.

We hereby report the unique immunophenotype of newly diagnosed APL early during ATRA therapy compared with untreated APL. Moreover, we compare these findings with cases of nonpromyelocytic AML (including HLA-DR– and/or CD34– cases) to evaluate the sensitivity and specificity of various flow cytometry diagnostic strategies in untreated and ATRA-treated APL. Our results underscore the importance of obtaining information regarding recent ATRA therapy when evaluating the flow cytometric findings of patients with presumptive APL.

**Materials and Methods**

**Patient Selection**

We identified patients with APL diagnosed between January 2005 and December 2013 who had flow cytometric analysis performed at our institution before first-induction chemotherapy. Scanned documents from referring institutions and clinical notes upon admission were reviewed to determine if the patient had received ATRA and for how long. As a control population, we included all other patients with newly diagnosed AML evaluated between January 2011 and December 2012. In addition, we also included all HLA-DR– AMLs evaluated at relapse during the same period to obtain a more representative population of this infrequent immunophenotype. This study was approved by the University of South Florida Institutional Review Board.

**Flow Cytometry**

Aliquots of 50 µL whole blood in sodium heparin were incubated in the dark for 15 minutes at room temperature with combinations of four monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein–cyanine 5.5 (PerCP-Cy5.5), or allophycocyanin (APC). RBCs were lysed with BD FACS lysing solution (BD Biosciences, San Jose, CA), and nucleated cells were resuspended in phosphate-buffered saline containing 2% paraformaldehyde. At least 200,000 events were acquired on a FACSCalibur flow cytometer (BD Biosciences). A four-color antibody panel (FITC/PE/PerCP-Cy5.5/APC) was performed, consisting of a modified isotype control tube (including CD45-PerCP-Cy5.5) plus six tubes (CD14/glycoporphin-A/CD45/isotype, CD7/CD10/CD45/ CD19, CD56/CD11b/CD45/CD11c, CD3/CD4/CD45/CD8, HLA-DR/CD117/CD45/CD33, and CD61/CD13/CD45/ CD34). In a few instances, a simplified four-color antibody panel was used, consisting of the modified isotype control tube plus three tubes (CD56/CD11b/CD45/CD11c, HLA-DR/CD117/CD45/CD33, and CD61/CD13/CD45/CD34). All antibodies were obtained from BD Biosciences.

Listmode files were retrospectively reanalyzed, based on ad hoc templates created on Kaluza, version 1.2 (Beckman Coulter, Brea, CA). In brief, a blast population was defined as the intersection of three interdependent polygonal gates drawn on three separate two-dimensional dot-plots, representing all six dimensions evaluable on a four-color tube, including forward scatter height (FSC-H) and side scatter height (SSC-H). Real-time color coding was used to visually narrow the polygonal gates to define clusters of cells with immunophenotypic properties expected for blasts. A separate three-dimensional blast gate based on FSC-H, SSC-H, and CD45 was created on the most informative tube and applied to the isotype control tube. Thresholds for FITC, PE, and APC positivity were defined to include only 2% of isotype-stained blast events. Positive expression was arbitrarily defined as equal to or greater than 20% of events with higher florescence intensity than the above-defined threshold.

**Statistical Analysis**

All statistical calculations were performed using GraphPad Prism, version 5.04 for Windows (GraphPad Software, San Diego, CA). Differences between percentages of positive events were calculated using the Mann-Whitney rank sum test. A statistically significant \(P\) value was considered as less than .05.
Results

Twenty-nine patients with newly diagnosed APL were evaluated at Moffitt Cancer Center (MCC) between 2005 and 2013. Chromosomal analysis showed t(15;17) in 20 patients, t(11;17) in two patients, and a tetraploid karyotype in one patient (cryptic PML-RARA translocation), all of whom were confirmed to harbor an RARA gene translocation by fluorescence in situ hybridization analysis (FISH). In the remaining six patients in whom a chromosomal analysis was not available, APL was confirmed by FISH for PML-RARA (three patients), FISH for RARA translocation (two patients), or polymerase chain reaction for PML-RARA (one patient). Three patients exhibited a microgranular variant morphology (microgranular APL). The median age was 53 years (range, 22-77 years), and the male to female ratio was 1.2:1. Flow cytometry was performed on bone marrow aspirates in 24 patients and on peripheral blood in five patients. Of all patients with APL, 17 (59%) had already received one or more doses of ATRA, starting a median of 1 day (range, 1-8 days) before obtaining a specimen at the MCC for flow cytometry.

An expanded blast population was identified by flow cytometry in all patients with APL, accounting for a median of 74% of total events (range, 19%-95%). As expected, most were positive for CD117 and negative for CD34 and HLA-DR ❚Figure 1❚, including both patients with the t(11;17) variant translocation and all three patients with microgranular APL. There were no significant differences in the percentage of blast events positive for CD34, CD117, or HLA-DR when comparing patients with or without recent ATRA therapy ❚Figure 2a. Interestingly, all 12 patients with APL who did not have prior ATRA therapy showed very low numbers of blast events positive for CD11b or CD11c (median, 3.3% and 2.3%, respectively), which were always below the 20% threshold for positivity. In sharp contrast, patients with APL who were recently treated with ATRA showed a much higher percentage of CD11b- and CD11c-positive events (median, 49.2% and 58.7%, respectively; P < .0001 for both), which in most cases was over 20% (Figures 1 and 2). Similarly, the percentage of CD4-positive blast events was significantly higher in patients previously treated with ATRA (median, 38.2%) compared with patients with APL who did not have prior ATRA administration (median, 7.4%; P = .0126). There were no significant differences in the expression of CD10, CD13, CD14, CD33, or CD56 between ATRA-treated and untreated APLs (Figure 2).

On the basis of the documented date of ATRA therapy, we observed expression of CD11b and CD11c above the 20% threshold as early as 1 day after ATRA administration ❚Figure 3a. After day 3 of therapy, all patients showed positivity for CD11b and CD11c, suggesting a time dependence trend in the upregulation of these markers. Expression of CD4 after ATRA therapy was haphazard and did not appear to be related to the number of days on therapy.

Using similar gating strategies, we studied the immunophenotype of 93 patients with nonpromyelocytic AML. The median age was 60 years (range, 23-89 years), and the male to female ratio was 1.1:1. Chromosomal analysis
showed a normal karyotype in 44 patients, myelodysplasia-related abnormalities (including complex karyotypes) in 24 patients, nonrecurrent chromosomal abnormalities in nine patients, inv(16)(p13.1q22) in six patients, t(9;11)(p22;q23) in three patients, other 11q23(MLL) translocations in four patients, t(8;21)(q22;q22) in two patients, and inv(3)(q21q26.2) in one patient. On the basis of cytomorphologic features, patients were classified into the following categories: M0 (n = 8), M1 (n = 21), M2 (n = 24), M4 (n = 19), M5 (n = 17), or M6 (n = 4). Flow cytometry was performed on bone marrow aspirates in 73 patients and on peripheral blood in 20 patients.

When comparing percentages of positive blasts above a standardized threshold, both ATRA-treated and untreated APL exhibited distinctively low numbers of CD34+ and HLA-DR+ events compared with most nonpromyelocytic AMLs. In our control population, 25 (27%) of 93 patients were negative for CD34 or HLA-DR, and five (5.4%) were negative for both. As a group, these patients could be distinguished from untreated APL by their frequent expression of CD4, CD11b, and CD11c. In contrast, the immunophenotype of ATRA-treated APL showed close resemblance to CD34– or HLA-DR– nonpromyelocytic AMLs, except for a higher frequency of CD36 expression in the latter. Other features of APL, such as expression of CD117, CD13, and CD33, were also encountered in most other AMLs, thus limiting their use as diagnostic cues (Figure 4).

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Next, we compared the diagnostic utility of single-surface antigens or antigen combinations before and early during therapy with ATRA. Interestingly, a combination of the three best-known immunophenotypic features of APL (CD34–, CD117+, and HLA-DR–) showed low diagnostic sensitivity in both untreated (67%) and ATRA-treated (59%) APL. By themselves, both CD34– and HLA-DR– identified the majority of patients with APL independently of prior ATRA therapy, achieving 100% sensitivity but suboptimal specificity (73%) when combined in a nonexclusive fashion (CD34– or HLA-DR–). The combination of CD11c– and CD11b– was 100% sensitive and 87% specific for the
identification of untreated APL but showed no diagnostic utility early during ATRA therapy. Absence of both CD11b and CD11c, in combination with either absent CD34 or HLA-DR, showed the best sensitivity (100%) and specificity (98%) for diagnosing APL in patients who had not started ATRA therapy.

Discussion

APL has a characteristic immunophenotype that facilitates its identification by flow cytometry. Nevertheless, not all APL-associated features are present in every case. CD34, for example, is reported to be expressed in 20% to 40% of APLs, particularly in the microgranular variant. Conversely, other types of AMLs can occasionally exhibit an immunophenotype reminiscent of APL. In particular, lack of HLA-DR expression has been described in a minority of nonpromyelocytic AMLs, with or without CD34 expression.

Further complicating this immunophenotypic variability is the fact that ATRA can sometimes be emergently initiated before comprehensive immunophenotypic analysis, based on characteristic peripheral blood morphology and clinical features at presentation. ATRA signals through the RAR family of nuclear receptors, resulting in reversion of the PML-RARA–enforced transcriptional control and maturation of APL blasts toward terminally differentiated granulocytes. In vitro, ATRA-induced differentiation has been shown to result in marked immunophenotypic changes in cell lines and primary APL blast cultures. Yet, the specific in vivo immunophenotypic changes of APL after ATRA administration have rarely been studied, and the implications for the flow cytometric diagnosis of APL have not been specifically addressed. In this study, we characterize the
unique immunophenotype of APL early during ATRA therapy and assess the diagnostic utility of flow cytometry in untreated and ATRA-treated APL.

Similar to recent studies,\textsuperscript{10,12} we show that evaluation of only CD34, CD117, and HLA-DR expression results in a suboptimal test performance for the flow cytometric identification of APL, underscoring the need to assess additional informative antigens. We and others have previously demonstrated that APL (without prior therapy) characteristically lacks expression of the β2-integrins CD11a, CD11b, CD11c, and CD18.\textsuperscript{20,22} More recently, lack of both CD11b and CD11c was found in all APLs studied but only 2% of nonpromyelocytic AMLs.\textsuperscript{10} In our study, we found absent CD11b and CD11c in all 12 untreated APLs but in only 13% of nonpromyelocytic AMLs. Our results support the integration of CD11b and CD11c in the flow cytometric diagnostic criteria for APL when evaluating patients who have not received ATRA therapy.

In our series, 59% of patients with APL had already received at least one dose of ATRA before diagnostic workup at our institution. We report that the characteristic expression pattern for HLA-DR, CD117, and CD34 in APL is largely preserved early during ATRA therapy. Nevertheless, CD11b and CD11c expression is sharply upregulated as early as 1 day after initiation of ATRA therapy. These findings are consistent with the documented upregulation of β2-integrins in primary APL cultures\textsuperscript{19,20} and APL-derived cell lines\textsuperscript{21} after in vitro treatment with ATRA. In a separate cohort of patients, we previously reported a rapid in vivo upregulation of β2-integrins after ATRA administration when studying sequential flow cytometry analyses before and during therapy.\textsuperscript{22} Both our prior and current studies suggest a greater effect on β2-integrin expression with increased number of days on therapy but a marked variability among individual cases. Given the rapid upregulation of β2-integrins observed in some patients, expression of CD11b and CD11c by flow cytometry should be interpreted with extreme caution in patients who had recently received ATRA for presumptive APL, irrespective of the number of days on therapy or doses received.

In our series, the best flow cytometric strategy for the diagnosis of APL in patients early during ATRA (lack of CD34 or HLA-DR expression) resulted in 100% sensitivity but a disappointing 73% specificity. Two other β2-integrins not included in our panel, CD11a and CD18, have shown to be characteristically absent in most APLs but expressed in most other AMLs.\textsuperscript{11,12} Interestingly, in vitro treatment of APL with ATRA has been shown to induce expression of CD18\textsuperscript{23} but not CD11a.\textsuperscript{19} If these results are demonstrated in vivo, lack of CD11a could be used in the diagnosis of APL irrespective of prior ATRA administration.

This retrospective study has some limitations that warrant caution in the interpretation of results. First, the exact dose and number of doses of ATRA could not be ascertained based on the limited information provided by the referring institutions, thus precluding a dose-dependent analysis of the in vivo immunophenotypic effect of ATRA on APL blasts. Also, we did not encounter cases of nonpromyelocytic AMLs treated with ATRA for presumptive APL, which would have contributed to a more strict evaluation of specificity. Finally, antibody combinations, gating strategies, and determination of antigen positivity in flow cytometry are not standardized, precluding direct comparisons with results from other laboratories. Despite these limitations, our results show clear-cut immunophenotypic differences when applying reproducible and strict flow cytometric criteria that minimize subjectivity in the interpretation of flow cytometric findings.
In this study, we demonstrate the unique immunophenotype of APL early during therapy with ATRA, in comparison to untreated APLs and nonpromyelocytic AMLs. Moreover, we describe the diagnostic utility and limitations of flow cytometry in patients on ATRA therapy. Careful evaluation of the clinical history for recent ATRA administration is essential for the appropriate interpretation of flow cytometric findings in patients with presumptive APL.

References


