Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

MIFlowCyt

Consistent Quantitative Gene Product Expression: #3. Invariance with Age

Michael, R. Loken,* Andrew P. Voigt, Lisa Eidenschink Brodersen, Wayne Fritschle, Andrew J. Menssen, Denise A. Wells

HematoLogics, Inc, Seattle, Washington

Received 13 June 2016; Accepted 24 September 2016

Additional Supporting Information may be found in the online version of this article.

Conflict of interest: A.P.V., L.E.B., W.F., and A.J.M. are employed by HematoLogics, Inc. M.R.L. and D.A.W. are equity owners of HematoLogics, Inc.

*Correspondence to: Michael R. Loken, HematoLogics, Inc., 3161 Elliot Ave, Suite 200, Seattle, WA 98121. E-mail: mrloken@hematologics.com

Published online 00 Month 2016 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.22997

Abstract

The quantitative expression of cell surface antigens and light scattering properties of five cellular reference populations in stressed bone marrow specimens were compared between pediatric and adult patients treated for acute myeloid leukemia (AML). The mean intensity of each antigen as well as the within patient and between patient variability showed striking consistency between the two different age groups. The only difference between the groups of specimens was the proportion of progenitor cells in the adult cohort averaged less than three times the proportion in the pediatric cohort. These data show that the amounts of gene products expressed on bone marrow cells are invariant with age. © 2016 International Society for Advancement of Cytometry

Key terms

flow cytometry; bone marrow aspirates; adult; quantitative antigen expression; support vector machines

Introduction

A companion manuscript identified five key reference populations that could be routinely detected in regenerating bone marrow aspirate specimens from pediatric patients treated on a single clinical protocol early post chemotherapy for acute myeloid leukemia (AML) (1). The data demonstrated that the amounts of gene products were highly regulated, with many gene products exhibiting minimal variability within individuals and even less variability between individuals. In this manuscript we extend this analysis to adults recovering from chemotherapy for AML.

Materials and methods

Patient Data Set

A total of 50 randomly selected, adult acute myeloid leukemia patients (age 23–73, median = 55.5) obtained post chemotherapy were identified as having no evidence of residual disease (2). Patients eligible for this study satisfied three criteria: (1) a prior history of treatment for AML, (2) submission of a normal, regenerating bone marrow aspirate without detectable residual disease by flow cytometry, and (3) evidence of high specimen quality with minimal hemodilution (3). In contrast to the pediatric specimens described in the companion manuscript, the bone marrow specimens from the adults were not part of a clinical study but were submitted for monitoring response to therapy in patients with AML. Therefore, the specimens were more heterogeneous not only with respect to age of the individual, but also were not standardized to a single therapy regimen nor to a single time point post therapy. Adult specimens from both pre- and post-hematopoietic stem cell transplant therapy were included in this study. This study was conducted following the guidelines of the Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects.

ISAC
International Society for Advancement of Cytometry

Table 1. Comparison between pediatric and adult values for CD45 and log Side Scatter (SSC) for 5 reference bone marrow cell populations

	PEDIATRIC CD45	ADULT CD45	PEDIATRIC SSC	ADULT SSC
Lymphocytes				
parameter mean	2.79	2.81	1.24	1.31
between patient variation	0.085	0.074	0.071	0.086
within patient variation	0.11	0.13	0.14	0.13
replicate variation (n=8)	0.017	0.018	0.02	0.024
Uncommitted Progenitors				
parameter mean	1.97	1.99	1.46	1.52
between patient variation	0.094	0.098	0.09	0.13
within patient variation	0.17	0.19	0.17	0.18
replicate variation ($n = 8$)	0.032	0.039	0.053	0.074
Promyelocytes				
Parameter mean	2.04	2.06	2.43	2.38
Between patient variation	0.097	0.080	0.054	0.089
Within patient variation	0.11	0.12	0.14	0.14
Mature Neutrophils				
Parameter mean	2.40	2.46	2.32	2.32
Between patient variation	0.18	0.19	0.076	0.098
Within patient variation	0.29	0.29	0.17	0.19
Mature Monocytes				
Parameter mean	2.76	2.82	1.86	1.92
Between patient variation	0.088	0.080	0.055	0.083
Within patient variation	0.16	0.15	0.15	0.15

Specimen Collection

Bone marrow aspirates were collected in heparin (the preferred anti-coagulant) or EDTA. The data were obtained over a period of 2 years and 6 months (overlapping in time with the analysis of the specimens for the pediatric study) using three separate flow cytometers, multiple reagent lots and processed by multiple technicians.

Flow Cytometry

Specimens were processed as routine clinical bone marrow aspirates as previously described (2). Briefly, 100 μL of bone marrow was added to cocktails of pre-tittered antibodies at room temperature in the dark. Red blood cells were lysed using 3.5 mL of buffered NH_4Cl (0.83%) at 37°C for 5 min before centrifugation at 300G. Cells were then washed with 3 mL of phosphate buffered saline containing 2% fetal calf serum and re-suspended to 0.5 mL in 1% paraformaldehyde for analysis on one of three FACS Calibur instruments (Becton Dickinson Biosciences, San Jose, CA). 200,000 events were collected for each tube. The flow cytometers were cross standardized and calibrated using RCP-30A and RFP-30A beads (Spherotech, Lake Forest, IL) with spectral compensation performed using peripheral blood cells labeled with CD4 (SK3, BD) conjugated to fluorescein (FITC), phycoerythrin (PE),

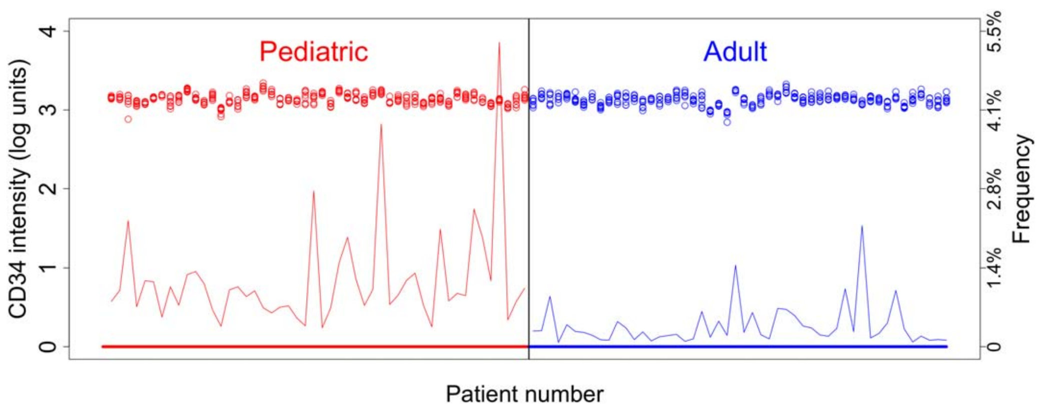

Figure 1. Comparison of the intensity of CD34 on the uncommitted progenitor cells between pediatric patients (red) and adult patients (blue). Replicate evaluations (8) for each patient are also displayed. The frequency of the uncommitted progenitor cells for the pediatric and adult groups are depicted as a line on the lower part of the graph, with frequency measurements scaled according to the y-axis on the right side of the plot.

Table 2. Intensity of CD34 on uncommitted progenitor cells is the same between pediatric and adult patients

UNCOMMITTED PROGENITORS	PEDIATRIC CD34	ADULT CD34
Parameter mean	3.14	3.13
Between patient variation	0.065	0.066
Within patient variation	0.15	0.15
Replicate variation $n = 8$	0.032	0.034

Table 4. Mature neutrophils express the same intensity of CD16 and CD13 in pediatric and adult patients

MATURE NEUTROPHILS	CD16 PED	CD16 ADULT	CD13 PED	CD13 ADULT
Parameter mean	3.07	3.11	2.81	2.79
Between patient variation	0.11	0.11	0.15	0.20
Within patient variation	0.16	0.18	0.26	0.26

peridinin chlorophyll protein (PerCP) or allophycocyanin (APC). Eight combinations of antibodies were used as previously described (1,4).

Support Vector Machines

Support vector machines (SVMs) were trained on 27 pediatric bone marrow specimens from patients recovering from chemotherapy (1,4). These same SVMs were used to analyze the adult patients. The time period for acquisition of these data overlapped that of the pediatric group with data collected over greater than a 2-year period. A more detailed description of the analytic approach is contained in a companion article (4).

Results

The same five reference populations could be identified in all adult specimens as in the pediatric study: (1) Mature lymphocytes, identified by high CD45 and low log SSC, were detected in all eight reagent tubes, (2) Uncommitted progenitor cells, identified by high expression of CD34 and intermediate CD33, were detected in all eight reagent combinations, (3) Promyelocytes, identified by high log SSC without expression of HLA-DR or CD11b, (4) Mature monocytes, identified by high expression of CD33 and CD14, and (5) Mature neutrophils, identified by high expression of CD16 and CD13.

The parameter means and variation characteristics of CD45 and log SSC were determined for all five adult populations and compared with the results from the pediatric study (Table 1). The CD45 and log SSC parameter means for all five reference populations were indistinguishable between the pediatric and adult patients. Likewise, the CD45 and log SSC replicate variation characteristics were very similar between the pediatric and adult populations. Notably, the within-patient, between-patient, and replicate variation were slightly elevated for the uncommitted progenitor cells in the adult group. This small increase could only be observed by statistical analysis and could not be detected by visually comparing the populations on a four-decade log scale. The between patient variability, i.e. variability of the means between patients, was always less than the within patient variability for both the adult and pediatric groups for CD45 and log SSC.

CD34 expression on the uncommitted progenitor cells was compared between the pediatric and adult patients (Fig. 1). The parameter means for both the pediatric and the adult were essentially identical (3.14 vs. 3.13 log units) (Table 2). The replicate variability between the eight tubes for CD34 intensity was the same between the pediatric and adult Group (0.032 vs. 0.034 log units) (Table 2). The within patient variation (0.15 vs. 0.15 log units) as well as the between patient variability (0.065 vs. 0.066 log units) was also the same in pediatric and adult patients. The major difference between pediatric and adults was observed in the total number of uncommitted progenitor cells (Fig. 1). The proportion of total events identified as uncommitted progenitor cells in the pediatric group (mean = 1.2% of total cellular events) was >3 times higher as compared to the proportion of total events identified as uncommitted progenitor cells within the adult group (mean = 0.35% of total cellular events).

A comparison of the gene products expressed on mature monocytes demonstrated that the highly regulated antigens CD14, CD33, and CD64 demonstrated similar mean intensities, within patient variation, and between patient variation for both pediatric and adults (Table 3). CD36 expression is broader in both pediatric and adults and again demonstrated similar means intensities, within patient variation, and between patient variation. In both patient populations, the between patient variation for CD33 was also greater than the within patient variation.

The mature neutrophils, identified by high expression of CD16 and CD13, also demonstrated reproducible results comparing pediatric and adult patient populations (Table 4). The differences could only be identified using statistical analysis and could not be seen in visual inspection on a four-decade (log) scale. As shown for the pediatric group, CD16 demonstrates a tighter within patient variability than CD13 for the adults. Therefore, the position of the mature neutrophils with respect to CD13 and CD16 is the same between pediatric and adult patients.

Table 3. Mature monocytes express the same intensity of CD14, CD33, CD36 and CD64 in pediatric and adult patients

MATURE MONOCYTES	CD14 PEDIATRIC	CD14 ADULT	CD33 PEDIATRIC	CD33 ADULT	CD64 PEDIATRIC	CD64 ADULT	CD36 PEDIATRIC	CD36 ADULT
Parameter mean	2.54	2.53	2.74	2.82	2.88	3.01	2.99	2.99
Between patient variation	0.062	0.082	0.25	0.27	0.11	0.11	0.21	0.22
Within patient variation	0.18	0.17	0.14	0.13	0.18	0.21	0.33	0.28

Discussion

The data presented in this manuscript extend the study of constancy of gene product expression identified in stressed bone marrow specimens from pediatric to adult patients. The adult patients were not standardized to a single chemotherapeutic regiment nor were they standardized to a specific time after chemotherapy as specified in the pediatric study. In fact, many of the adult patients were assessed following a hematopoietic stem cell transplant.

In normal bone marrow, hematopoiesis is characterized by reproducible changes in the quantitative expression of surface gene products, independent of age or marrow stress. Infant, adolescent, adult, and elderly patients exhibit identical phenotypic patterns on the investigated reference populations, even during the administration of chemotherapy and after bone marrow transplants. Taken together, these data illustrate that the quantitative amount of surface gene product expression is a biologic constant, suggesting that in order for a cell to properly mature, the amounts of surface antigens must be precisely regulated.

Although intensity relationships are identical between individuals, the proportion of cells in various maturational stages may vary. The adult population had a substantially decreased proportion of events identified as uncommitted progenitor cells compared to the pediatric population. These data suggest that the adult population may have a distinct kinetic response to chemotherapy, regenerating progenitor cells at a slower rate than the pediatric population. The decreased number of events in this minor population can increase the variability in calculating the mean and variation characteristics of this small population, and may be responsible for the observed increase in replicate and between patient variability in CD45 and log SSC for the uncommitted progenitor cells.

The data presented in this manuscript demonstrate a consistency in cellular gene product expression as well as cellular characteristics such as log SSC. Although the proportions of different cell populations can vary, the amounts of gene products expressed at specific stages of development are identical between the pediatric and adult group and demonstrate a high level of regulation in these key cellular characteristics. These data indicate that during the maturational process the amounts of gene products expressed are identical between pediatric, adult and even elderly patients. It appears that quantitative gene product expression is an inherent property of maturation of each cell type, independent of age and marrow stress. The mechanism controlling this tight gene product regulation has yet to be elucidated.

Literature Cited

1. Loken MR, Voigt AP, Brodersen LE, Fritschle W, Menssen AJ, Meshinchi S, Wells DA. Consistent quantitative gene product expression: #2. Antigen intensities on bone narrow cells and invariant between individuals. Cytometry A 2016; (in press). DOI: 10.1002/cyto.a.22999.
2. Loken MR, Alonzo TA, Pardo L, Gerbing RB, Raimondi SC, Hirsch BA, Ho PA, Franklin J, Cooper TM, Gamis AS, et al. Residual disease detected by multidimensional flow cytometry signifies high relapse risk in patients with de novo acute myeloid leukemia: A report from Children's Oncology Group. Blood 2012;120: 1581–1588.
3. Loken MR, Chu SC, Fritschle W, Kalnoski M, Wells DA. Normalization of bone marrow aspirates for hemodilution in flow cytometric analyses. Cytometry B 2008;76B: 27–36.
4. Voigt AP, Brodersen LE, Pardo L, Meshinchi S, Loken MR. Consistent quantitative gene product expression. #1. Automated identification of regenerating bone marrow cell populations using support vector machines. Cytometry A 2016; (in press). DOI: 10.1002/cyto.a.22905.