Detection of endogenous alkaline phosphatase activity in intact leukemic cells

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ABSTRACT

With the aim to detect candidate malignant primitive progenitor populations in human leukemia cells, we modified the original Alkaline Phosphatase (ALP) stem cell detection method based on the identification of alkaline phosphatase fluorescent cells in combination with flow cytometry immunophenotyping. Preliminary data obtained in our laboratory have shown that ALP can be expressed at high levels in leukemia. By using these newly developed panels, leukemic cells can be classified into different ALP functional states. Our results suggest that the main differences in the activity of the enzyme could help to identify and differentiate new populations in patients with neoplastic malignancies.

INTRODUCTION

Alkaline phosphatase (ALP) is a 140 kD enzyme with a dimeric structure and is capable of binding Zn^{2+} and Mg^{2+} ions at different sites to stimulate or inhibit its catalytic reaction. In 1973, ALP was reported as a histochemical marker for embryonal carcinoma cells with two different alkaline phosphatases localized in stem cell populations and in embryonic ectodermal cells. ALP is used as a marker for pluripotent stem cells, embryonic stem cells, induced pluripotent stem cells and embryonic germ cells. ALP is highly elevated in these cells and western blot, ELISA, and chromogenic substrates in combination with immunohistochemistry are commonly used to assay these levels. More recently, alkaline phosphatase activity has been analyzed using highly sensitive fluorescent and chemiluminescent substrates, in both fixed and living cells.

With the aim to detect candidate malignant primitive progenitor populations in human leukemia and lymphoma cells, we modified the original ALP stem cell detection method based on the identification of alkaline phosphatase fluorescent cells in combination with flow cytometry immunophenotyping.

MATERIALS AND METHODS

Peripheral blood and bone marrow samples from leukemia patients were studied at diagnosis, for minimal residual disease monitoring, and relapse. ALP staining was combined with immunophenotyping and no-lyse no-wash methods using the Invitrogen™ Attune™ NxT Flow Cytometer (Thermo Fisher Scientific). Anticoagulated blood or marrow containing $5x10^5$ cells was diluted 1/10 in HBSS, and 100 µL of diluted blood was used for incubation for 20 min at 37ºC in the presence of 1 µL of APLS and 1 µL of Vybrant™ DyeCycle™ Violet (DCV) stain, protected from light in a dedicated water bath. Following incubation 5 µL of the corresponding antibodies were added to cells, and incubated again for 20 min at room temperature, also protected from light.

RESULTS AND DISCUSSION I

We have been using this protocol to study the alkaline phosphatase activity in human leukemia and lymphoma. Here we show one-year history from a follow-up of the same patient, a 27-year-old female diagnosed in May 2010 with B common acute lymphoblastic leukemia (Case 1). Antigen expression was evaluated at first relapse (November 2012), showing 40% CD34+ blast cells with identical phenotype of refractory disease as compared with diagnosis. On February 2015, the patient had a second relapse with 88% of blast cells. On February 2016 the patient had a third cytological relapse (82% of blast cells with B-ALL phenotype). She finally died on April 3, 2016, 6 years after diagnosis. Five bone marrow aspirate specimens were taken from May 2015 to April 1st 2016 (Figure 1). By using the alkaline phosphatase assay, our results suggest that apparently clonal leukemic refractory CD34+ cells can be classified into functional states based upon the different activity levels of the phosphatase. If true, the main differences in the activity of the enzyme, accordingly with previous observations showing that primitive stem cells express the highest phosphatase activity, could help to identify and differentiate new oligoclonal/pseudoclonal populations in patients with neoplastic malignancies. Preliminary results obtained in our laboratory have shown that alkaline phosphatase can be expressed at high levels in leukemia.

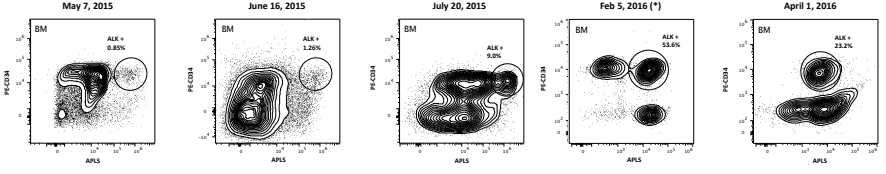

Figure 1. Highly refractory B-cell acute lymphoblastic leukemic CD34+ cells expressing high levels of alkaline phosphatase activity (Case 1). Reference contour plots for five bone marrow (BM) aspirates and one peripheral blood (PB) analysis obtained in the last year of the follow-up, showing the performance of the alkaline phosphatase test in combination with CD34 staining. Prospective comparison and classification of ALP+ cells from new independent bone marrow samples corresponding to the same B-ALL patient, show different subsets of CD34+ cells. Each individual circle represents CD34+ cells with high expressing levels of alkaline phosphatase activity, presumably enriched in primitive refractory cells. Comparison of well-defined encircled populations consisting of CD34+/ALP high cells provides valuable information for dual parameter contour plots over time, to better discriminate subsets of apparently clonal CD34+ cells.

RESULTS AND DISCUSSION II

We have observed that this activity is not always restricted to CD34+ leukemic cells but can be overexpressed in CD34 negative leukemic blasts from independent specimens (Figure 2). Figure 2A represents the ALP test of a bone marrow aspirate from a 61-year-old patient with myelodysplastic syndrome developed into acute myeloid leukemia at diagnosis (Case 2). Figure 2B represents a bone marrow aspirate from a 78-year-old patient with refractory anemia with excess blasts (RAEB-2) developed into acute myeloid leukemia at diagnosis (Case 3), and Figure 2C shows the APL test on a marrow aspirate from a 12-year-old patient with intermediate risk B-cell precursor acute lymphoblastic leukemia during maintenance therapy leaving an undetectable MRD (Case 4).

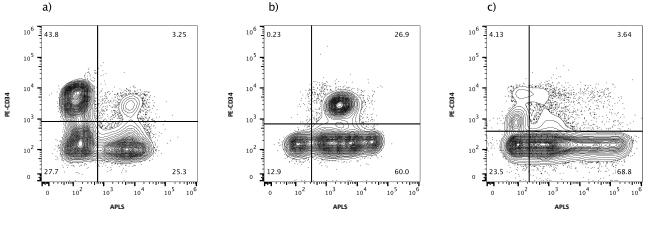

Figure 2. Alkaline phosphatase activity is not restricted to CD34+ cells. Figure (**A**) represents the alkaline phosphatase test of a bone marrow aspirate from a 61-year-old patient with myelodysplastic syndrome developed into acute myeloid leukemia. The time and date of sample collection was at diagnosis. Figure (**B**) represents a bone marrow aspirate from a 78-year-old patient with refractory anemia with excess blasts (RAEB-2) developed into acute myeloid leukemia at diagnosis, and Figure (**C**) shows the APL test on a marrow aspirate from a 12-year-old patient with intermediate risk B-cell precursor acute lymphoblastic leukemia during maintenance therapy leaving an undetectable minimal residual disease.

RESULTS AND DISCUSSION III

To evaluate the potential role of ALP+ cells in acute myeloid leukemia, we applied our methodology to a 43-year-old male (Case 5), with leukemic cells CD34+CD123+. Flow cytometry analyses of ALP activity in CD123+/CD34+ cells found in marrow show a differentiated ALP activity within the leukemic clone in two follow-up time points (Figure 3).

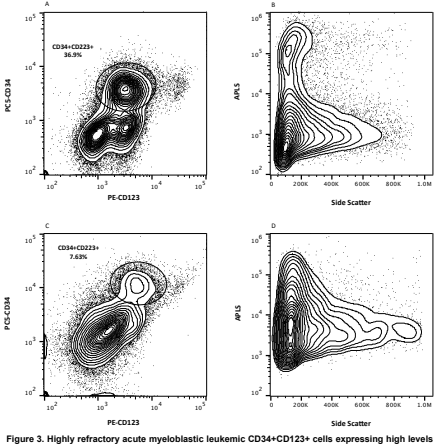

Figure 3. Highly refractory acute myeloblastic leukemic CD34+CD123+ cells expressing high levels of alkaline phosphatase at two time points during follow-up (Case 5). Representative contour plots for two marrow aspirates obtained from the fifth patient, displaying alkaline phosphatase activity in CD34+/CD123+ cells, showing timepoints of post-consolidation therapy (A,B) and pre-transplant evaluation (C,D). Prospective comparison and classification of ALP+ cells also shows different frequency and antigenic density of CD34+/CD123+ cells. Encircled cells represents CD34+/CD123+ populations with high expressing levels of alkaline phosphatase activity, presumably enriched in primitive leukemic refractory cells.

CONCLUSIONS

Our results suggest that the main differences in the activity of the ALP enzyme, accordingly with previous observations showing that primitive stem cells express the highest phosphatase activity, could help to identify and differentiate new oligoclonal/pseudoclonal populations in patients with neoplastic malignancies. We have verified that this method gives accurate and reproducible measurements and our preliminary results suggest that ALP^{high} leukemic cells appear to sustain leukemogenesis over time.