Single cell analysis of phosphoinositide 3-kinase/Akt and ERK activation in acute myeloid leukemia by flow cytometry

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Background and Objectives. Abnormal activation of several signal transduction pathways such as phosphoinositide 3-kinase (PI3K) and MAP kinases has been reported in acute myeloid leukemia (AML). To test new targeted therapeutics, it is critical to develop sensitive analytical tools to detect abnormal activation of these pathways and to monitor their inhibition in response to treatment.

Design and Methods. We analyzed Akt and ERK phosphorylation in 32 samples from patients using western blot and a two-color flow cytometry protocol using CD34. To circumvent the CD34 negative AML found in our series, we developed a two-color protocol using CD45 to isolate the blast cell population. Finally, a four-color protocol was used to detect phosphorylation in an enriched population of AML stem cells.

Results. We compared western blot analysis and flow cytometry for the detection of PI3K/Akt and ERK activation and found a 100% correlation between the two techniques in a series of 32 AML samples. Using a flow cytometry protocol, we were able to analyze all the patients' samples, even those with low blast infiltration or CD34 negative blast cells. We were also able to detect the phosphorylated proteins in the most immature blast cell population with the $CD34^{+}$ $CD38^{-/low}$ $CD123^{+}$ phenotype.

Interpretations and Conclusion. Our study shows that flow cytometry is a reliable method for detecting Akt and ERK phosphorylation in all patients' samples. Activation can also be detected in the most immature blast cells, which represent exquisite target cells for new therapeutics.

Key words: flow cytometry, PI3K/Akt, ERK kinase, phospho-protein, AML.

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Acute myeloid leukemias (AML) are clonal diseases due to acquired mutations in immature progenitors, resulting in a block of differentiation leading to an accumulation of immature myeloid blasts and oncogenic progression.[1] Two classes of mutations, one impairing cell differentiation and the other conferring cell survival and proliferative advantage, are known to cooperate to cause acute leukemia.[2] The phosphoinositide 3-kinase (PI3K)/Akt and mitogen activated protein kinase (MAPK) signaling pathways regulate many normal cellular processes including cell survival, growth and proliferation.[3,4] These two signaling pathways are also known as critical contributors of oncogenesis as they promote survival and proliferation of cancer cells.[5-7] The PI3K/Akt and MAPK pathways have been reported to be constitutively activated in leukemic blasts from patients with AML and to contribute to sustain proliferation, inhibit apoptosis and elicit transformation.[8-11] We have previously shown that the p110δ isoform of PI3K is consistently expressed at a high level in blast cells from patients with AML and is responsible for the constitutive activation of PI3K frequently observed in these blast cells.[12] The p110δ gene is not mutated in AML cells[13] and the mechanisms leading to the constitutive activation of p110δ remain to be identified. Recently, components of these pathways have emerged as promising targets for the development of specific inhibitory compounds. In particular, we showed that IC871,[14] a p110δ-selective inhibitor able to suppress Akt activation in AML blasts, inhibited the proliferation of AML cells with constitutive PI3K activation whereas it did not modify the proliferation of either normal hematopoietic progenitors or AML blast cells without detectable activation of PI3K.[12] The ERK pathway is also frequently activated in AML blasts and contributes to cell proliferation and inhibition of apoptosis.[14-16] Sensitivity of AML blasts to MEK inhibitors has been shown to correlate with detectable ERK activation since the proliferation of blast cells that expressed low or undetectable levels of ERK activity was not decreased by these inhibitors.[14] As targeted therapeutics are envisioned in AML treatment, a rapid analysis of the activation state of the main signaling pathways in blast cells from AML patient at diagnosis is required. The activation of the PI3K/Akt pathway is detected by analysis of the phosphorylation of the downstream Akt kinase on Ser 473,

whereas activation of the MAPK pathway is assessed by detection of ERK kinase phosphorylation. Analysis of phosphorylated proteins usually relies on western blot analysis but this technique has major limitations in AML: (i) the experiment is time consuming and high sensitivity western blot requires approximately 48-72 hours; (ii) a substantial number of cells is needed, which might not be available in several cases; (iii) in some AML subgroups, such as AML2, AML4 or AML5, the percentage of bone marrow blast cells is usually low and interpretation of western blots is clearly impossible.

Moreover, recent data suggest that the blast population is heterogeneous and that self-renewal capacity is restricted to a limited cell population considered as leukemic stem cells.[17] These leukemic stem cells are responsible for both self-renewal and production of clonogenic leukemic progenitors with proliferative potential.[18] Although some controversy remains concerning the phenotype of leukemic stem cells, these cells belong to a subpopulation of blasts that can be distinguished from most leukemic blasts by the expression of a set of cell surface markers. In particular, it has been shown that the leukemic stem cell population is typically comprised within the $CD34^+$, $CD38^-$, $CD71^-$, $HLA\text{-}DR^-$, $CD90^-$, $CD117^-$ and $CD123^+$ cell population.[19-21] Leukemic stem cells are rare and may be resistant to drugs designed to kill AML blasts. Thus, determining the relevance of abnormal activation of signaling pathways in this cell population is a very important direction for future research.

This study was initially designed to compare the detection of phosphorylated proteins of the PI3K/Akt and ERK pathways by western blotting and flow cytometry analyses in 32 newly diagnosed patients with AML and a high percentage of infiltrating blast cells. We determined the best antibody combination for flow cytometric investigation of the activation state of these two signaling pathways in AML blast cells, whatever the FAB subtype, the number of available cells for analysis, and the percentage of blast cells. Finally, we describe a four-color flow cytometry analysis that allows characterization of phospho-protein in very immature leukemic cells.

Design and Methods

Patients

All patients studied in this report were included in the AML2001 trial of chemotherapy initiated by the French Multicenter Group, Groupe Ouest-Est des Leucémies et des Autres Maladies du Sang (GOELAMS). All biological studies performed were approved by the GOELAMS institutional review board, and signed informed consent was provided according to the Declaration of Helsinki. The classification of the cases of AML was based on the French-American-British (FAB) criteria. Patients who presented with acute promyelocytic leukemia (AML3), erythroleukemia (AML6) and megakaryoblastic leukemia (AML7) FAB subtypes were excluded from the study. A total of 72 patients with *de novo* AML were included in this study.

Cells

Bone marrow or blood mononuclear cells (BMMC) from newly diagnosed patients were obtained before induction chemotherapy. The samples were subjected to Ficoll-Hypaque density gradient separation to isolate BMMC and red cells were lysed with ACK buffer (NH4Cl 155mM, KHCO3 10mM, EDTA 0.1mM, pH=7.4). The percentage of blast cells was evaluated by a May Grünwald Giemsa (MGG) staining of a cytospin. Before analysis, the cells were deprived of all growth factors by incubation for one hour in serum-free medium containing 0.1% deionized bovine serum albumin (BSA) and 25 μg/mL iron-loaded human transferrin in order to detect constitutive activation of the signaling pathways. Previous results showed that increasing the deprivation time up to 8 hours did not modify the observed level of Akt or ERK activation (*Bardet V, unpublished data*). In some control experiments, LY294002 (10 μM), an inhibitor of PI3K (Sigma, St Louis, MO, USA) and UO126 (10 μM), an inhibitor of ERK (Cell Signaling Technology), were added during the last 30 min of deprivation in order to inhibit phosphorylation. Normal bone marrow cells were obtained from patients undergoing hip surgery after informed consent. $CD34^+$ cells were isolated using a StemSep CD34 Human CD34 Positive selection kit (Cat. number 14756, Stem Cell Technologies, USA), according to the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed on freshly isolated cells, only from patients with more than 60% blast cell infiltration. Cells were boiled in Laemmli's sample buffer and proteins were analyzed by western blot. Briefly, proteins from 1×10^6 cells were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. The sheets were saturated in 5% non-fat dry milk phosphate-buffered saline (PBS, pH=7.4) for 60 min at room temperature, then incubated overnight at 4°C in 5% non-fat dry milk PBS containing the primary antibodies to Ser 473 p-Akt (Cat. number 9271, Cell Signaling Technology, Beverly, MA, USA) and to Thr 202/Tyr 204 p-p42/p44 MAPK (Cat. number 9101, Cell Signaling Technology). After five washes in PBS containing 0.1% Tween 20, they were incubated for 45 min at room temperature with anti-rabbit peroxidase conjugate secondary antibody (Cat. number 7074, Cell Signaling Technology) diluted 1/10 000 in 5% non-fat dry milk PBS-Tween 20, and washed as described above. SuperSignal West Femto (Pierce, Rockford, IL, USA) chemoluminescence kits were used for detection. To ensure equal loading, blots were probed with a mouse monoclonal antibody to β actin (Cat. number A 5441, Sigma). The efficiency of p-ERK and p-Akt detection was checked in each western blot experiment by using an extract of erythropoietin-stimulated UT7 cells.[4]

Intracellular staining and flow cytometry analysis

Cells of patients with AML ($1\text{-}5\times10^5$/sample) were resuspended in PBS and stained according to three different protocols to detect activation of the PI3K/Akt and ERK pathways in blast cells.

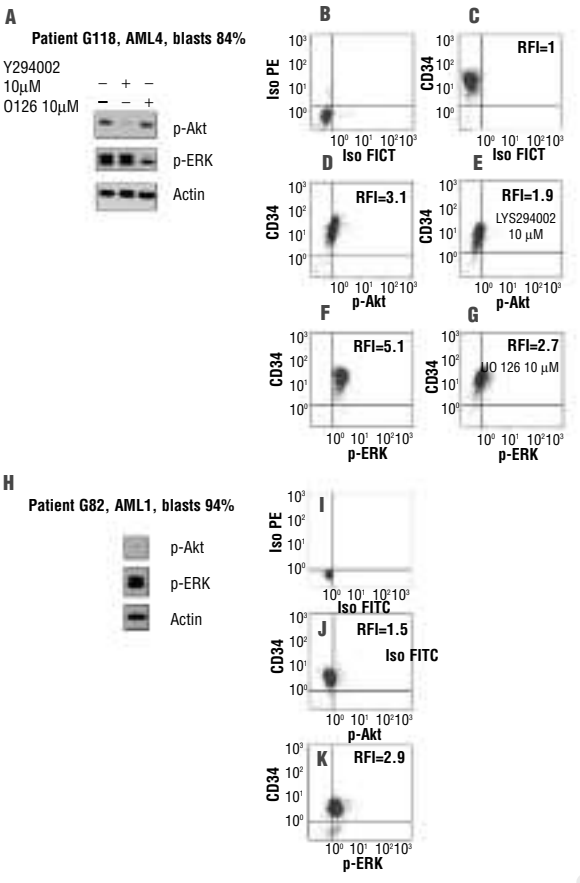

Figure 1. Correlation between western blot and flow cytometry in AML samples from patients G118 and G82. (A, H) Western blot analysis of samples from patients G118 and G82, respectively: 10 μM LY 294002 and 10 μM UO126 were used to inhibit p-Akt and p-ERK activation, respectively. (B-G, I-K) Flow cytometric analysis of samples from patients G118 and G82, (B,I) isotypic controls, (C) RFI of the cells stained with CD34-PE and FITC isotypic control, (D,J) CD34 and p-Akt expression, (E) effect of 10 μM LY294002, (F,K) CD34 and p-ERK expression, (G) effect of 10 μM UO126. RFI are indicated for the p-Akt and p-ERK staining.

Double staining with CD34: cells were incubated with PE-conjugated anti-CD34 (CD34 PE, Cat. number IM1871, Beckman Coulter, Miami, FL, USA) at room temperature for 15 min according to the manufacturer's instructions.

Double staining with CD45: cells were incubated with PC5-conjugated anti-CD45 (CD45 PC5, Cat. number IM2653, Beckman Coulter) at room temperature for 15 min.

Quadruple staining with CD38, CD34 and CD123: cells were incubated with PC7 conjugated anti-CD34 (CD34 PC7, Cat. number A07509, Beckman Coulter), PE-conjugated anti-CD38 (CD38 PE, Cat. number A07779, Beckman Coulter) and PC5 conjugated anti-CD123 (CD123 PC5, Cat. number 551065, Pharmingen, San Diego, CA, USA) at room temperature for 15 min.

Cells were then fixed with reagent 1 of the Intraprep kit (Cat. number IM2389, Beckman Coulter) for 15 min and washed with PBS. Cells were permeabilized with saponin-based reagent 2 and incubated for 45 min with a 1/100 dilution of antibody to Ser 473 p-Akt (Cat. number 4058, Cell Signaling Technology), or 1/50 dilution of antibody to Thr 202/Tyr 204 p- p44/p42 MAPK (Cat. number 9101, Cell Signaling Technology), or polyclonal rabbit immunoglobulins (Cat. number I8140, Sigma) as isotypic control. Samples were washed with PBS and pellets were incubated with 30 μL of 1/100 dilution FITC-conjugated goat anti rabbit F(ab)' IgG (Cat. number F1262, Sigma) for 30 min. Samples were washed with PBS, the pellets resuspended in 500 μL PBS and analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with CXP software.

p-Akt and p-ERK expression was analyzed and compared with that of the isotypic control. We analyzed the ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of the stained cells and the MFI of the isotypic control. Cells were considered as positive if the RFI ≥2 (Gaussian distribution), thereby allowing to the series of patients to be divided into two non-overlapping groups.

Results

Correlation between western blot and intracellular phospho-specific flow cytometry for the detection of PI3K/Akt and ERK activation

Before using flow cytometry analysis as a routine technique to detect the activation of the PI3K/Akt and ERK pathways in AML samples, we compared the results of flow cytometry with those of the reference technique, which is western blot analysis of phosphorylated proteins. For this purpose, we studied 32 patients with $CD34^+$ AML with a high percentage of bone marrow blast cells (mean 80%). Double immunostaining was performed to demonstrate the AML-associated antigen CD34 and phosphorylated Akt or phosphorylated ERK in leukemic cells. Representative results obtained in these experiments are reported in Figure 1. In the leukemic blasts of patient G118, constitutive activation of the PI3K and ERK pathways was detected by western blot, as assessed by phosphorylation of Akt and of ERK1/2 (Figure 1A). Akt phosphorylation was fully inhibited by the PI3K inhibitor LY294002 whereas ERK phosphorylation was partially inhibited by the MEK inhibitor UO126 (Figure 1A). Double staining a sample from the same patient (G118) with CD34PE and either FITC-conjugated p-Akt antibody or FITC-conjugated p-ERK1/2 antibody showed that the $CD34^+$ blast cells had Akt or ERK activation (RFI of 3.1 and 5.1, respectively). In this patient's sample, treatment with LY294002 or UO126 decreased Akt and ERK activation, respectively, as detected by western blot analysis and flow cytometry (Figure 1E and G). In the leukemic blasts of patient G82, Akt phosphorylation was not detected by western blot and the RFI for p-Akt was 1.5. In contrast, ERK phosphorylation was detected in this patient's cells by both techniques (Figure 1 H and K).

The results in this series of 32 patients are reported in Table 1. We found a 100% correlation between the two techniques for both phospho-Akt and phospho-ERK1/2

Table 1. **Main characteristics of the patients studied by western blot (WB) and flow cytometry (FC). Ratios of fluorescence intensity (RFI) are indicated for p-Akt and p-ERK staining in the CD34⁺ population.**

Patient	*FAB classification*	*Bone marrow Blast %*	*Cytogenetics*	*WB p-Akt*	*FC p-Akt/RFI*	*WB p-ERK*	*FC p-ERK/RFI*
G050	AML5	83	complex	+	+/2.1	+	+/2.2
G054	AML0	70	47, XX, +8	–	–/1	–	–/1.1
G055	AML1	67	46,XY; del 7(q21)	–	–/1.5	–	–/1
G061	AML2	90	46, XX	–	–/1	–	–/0.5
G069	AML4Eo	65	46,XX,inv(16)	+	+/2.9	+	+/3.5
G070	AML4Eo	66	46,XY, inv(16)	-	–/1	–	–/1
G072	AML5	60	46,XX,der(14)t(1;14),der(18)t(1;18)	+	+/2.3	+	+/2.6
G074	AML0	76	91,XXXX,-17	+	+/2.4	+	+/2.7
G079	AML4	62	46, XY	–	–/1.3	+	+/2.6
G082	AML1	94	46, XX	–	–/1.5	+	+/2.9
G089	AML1	65	46, XY,del(20q)	+	+/3.4	+	+/8.1
G093	AML4Eo	64	46,XY, inv(16), +8	–	–/1.5	–	–/1.5
G094	AML1	97	46, XY	–	–/1.5	–	–/1.3
G096	AML2	83	46, XX	–	–/1.2	–	–/1
G097	AML2	76	46, XX	+	+/2.0	+	+/3.8
G100	AML0	90	47, XY,+r(18)	+	+/2.2	+	+/3.5
G102	AML4	75	46, XY	+	+/2.1	+	+/3.7
G106	AML1	82	46, XY	+	+/2.3	+	ND
G107	AML2	80	46, XX	–	–/1.5	ND	ND
G115	AML1	96	46, XY, iso (17q)	–	–/1.3	–	ND
G117	AML2	64	46, XX, t(8,21),-X, del(2)(q32-33)	–	–/1.2	–	ND
G118	AML4	84	complex	+	+/3.1	+	+/5.1
G119	AML2	89	46,XX, t(8,21)	–	–/1	–	–/1.3
G120	AML5	98	82-89, complex	+	+/2.7	+	+/5.8
G135	AML1	100	46, XY	–	–/1	–	ND
G138	AML1	97	46, XX, del(5q), t(11;19), del(12p)	–	–/0.7	–	ND
G142	AML4	85	46, XX	+	+/3.0	+	+/5.7
G144	AML2	85	ND	–	–/1.2	+	+/3.3
G146	AML1	90	ND	+	+/2.0	+	+/6.4
G149	AML1	69	46, XY	–	-/1.1	–	–/1.2
G155	AML4	61	46, XX	+	+/3.8	+	+/4.5
G158	AML1	90	46, XY, del 7q	+	+/3.4	+	+/5.2

ND: not done. Cytogenetics revealing more than three abnormalities are indicated as complex.

detection. The global percentage of samples with constitutive PI3K/Akt activation was 47%. Twenty-six patients were studied by both western blotting and flow cytometry for ERK phosphorylation and constitutive activation of MAPK was found in 65% of them. As shown in Table 1, the RFI for Akt and ERK phosphorylation in samples positive by western blot analysis varied between patients, ranging from 2.1 to 3.8 for phospho-Akt and from 2.2 to 8.1 for phospho-ERK. In contrast, the RFI in samples found to be phosphorylation-negative using the western blot technique ranged from 0.7 to 1.5 for phospho-Akt and from 0.5 to 1.5 for phospho-ERK. Thus, the cut-off value of 2 clearly separated the positive and negative samples.

We analyzed the status of Akt and ERK phosphorylation in three normal bone marrow samples after CD34⁺ isolation. As shown in Figure 2 A-D, no phosphorylation of Akt or ERK was detected in these cells.

We then studied 20 additional CD34⁺ AML patients not evaluable by western blot analysis because of a small number of available cells ($<10^6$ cells) or a weak blast infiltration in the bone marrow (mean=41%). In these cases we used the double immunostaining flow cytometry technique and detected Akt phosphorylation in 45% (RFI: 2-3.4) and ERK phosphorylation in 70% (RFI: 2- 4.5) of AML blasts from these patients. We concluded that this intracellular phospho-specific flow cytometry technique could be used for rapid detection of activation of these two major signaling pathways in CD34⁺ AML.

AML samples can be studied for PI3K/Akt and ERK activation by gating the CD45^{low} blast cell population

Leukemic blast cells from patients with particular FAB subtypes, such as AML5 and AML2, often do not express the CD34 antigen. Indeed, 39% of AML samples analyzed in our institution were CD34-negative. We developed a flow cytometry technique allowing the pattern of activation of the PI3K and MAPK signaling pathways to be studied in all AML samples, whatever the CD34 phenotype of the leukemic population. The blast cell population can be identified by flow cytometry within the whole cell population by the level of CD45 expression and side scatter (SS). Blasts show a low level of CD45 expression and intermediate SS properties.[9,22] Results from two representative patients are shown in Figure 3. Patient G151 had a CD34⁻ AML4 with 30% blast cells in the blood. A dot plot of CD45 versus SS typically allowed identification of the

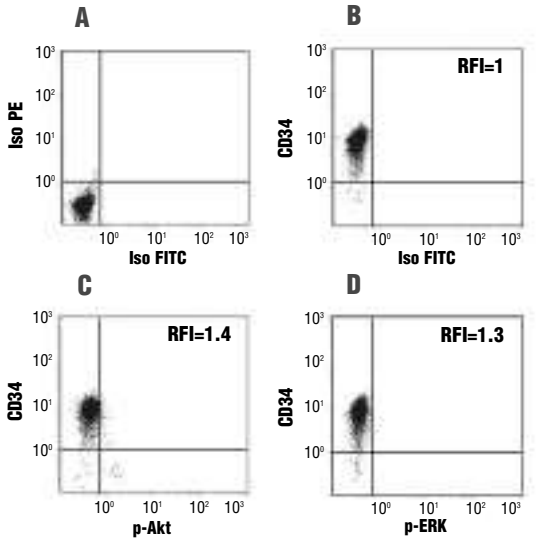

Figure 2. **Signal profile for p-Akt and p-ERK in normal bone marrow CD34⁺ cells. (A) isotypic controls, (B) cells stained with CD34-PE and FITC isotypic control, (C) cells stained for p-AKT, (D) cells stained for p-ERK. RFI are indicated on the dot plots.**

blast cell gate (B), the lymphocyte gate (C), the granulocyte gate (D) and the monocyte gate (E). When focusing on the blast population (gate B), we found that CD45^{low} cells had a RFI of 2.1 for phospho-Akt and of 3 for phospho-ERK (Figure 3C and 3E, respectively). In contrast, we did not detect any Akt and ERK phosphorylation in the lymphocyte population (gate C) (Figure 3D and 3F). In patient G153, who had a CD34$^-$ AML5 with 97% blast cells, no phosphorylation of Akt could be identified in the gate B CD45^{low} blast cells (RFI=1.2) (Figure 3I) whereas the blast population did show ERK phosphorylation (RFI=2.5) (Figure 3K). Lymphocytes (gate C) were negative for both

Table 2. **Main characteristics of the patients studied in the four-color protocol. RFI is indicated for the CD34⁺ population and for the CD34⁺ CD38$^{-/low}$ CD123⁺ population (called leukemic stem cells - LSC - in the table).**

Patient	*FAB classification*	*Bone marrow blast %*	*CD34⁺ %*	*CD34⁺ CD38$^{-/low}$ CD123⁺ %*	*p-Akt/RFI in CD34⁺*	*p-ERK/RFI in CD34⁺*	*p-Akt/RFI in LSC*	*p-ERK/RFI in LSC*
G123	AML0	60	61	33	–/1.3	ND	–/1.1	ND
G128	AML2	42	47	36	+/2.1	+/3.4	+/2.2	+/3.4
G135	AML1	100	38	5.2	–/1	ND	–/1	ND
G141	AML2	70	76	0.4	+/2.2	+/5.4	+/2.6	+/6.4
G145	AML2	80	85	0.1	+/3.1	+/5.8	+/3.7	+/6.0
G148	AML2	75	79	0.8	–/1.5	+/3.2	–/1.5	+/3.6

(Figure 3J and 3L). Fifteen patients with AML were analyzed using this two-color protocol. We found a complete correlation between the two labeling techniques using CD34 or CD45 in seven CD34⁺ patients. Thus, the status of intracellular p-Akt and p-ERK phosphorylation in the leukemic cells can be determined in AML by gating the CD45^{low} blast cell population.

Intracellular phospho-specific flow cytometry for PI3K/Akt and MAPK activation can be determined in a CD34⁺ CD38$^{-/low}$ CD123⁺ leukemic immature cell population by using four-color immunostaining

It is essential to characterize the properties of leukemic stem cells better for the future development of leukemia drugs, and especially for targeted therapies using inhibitors of signal transduction pathways. We attempted to analyze a population of AML stem cells defined by the CD34⁺, CD38$^{neg/low}$, CD123⁺ phenotype and to determine the status of intracellular Akt and ERK phosphorylation in this immature population. To this aim, bone marrow cells from six patients with CD34⁺ AML were stained with CD123 PC5, CD34 PC7 and CD38 PE, and after fixation and permeabilization,

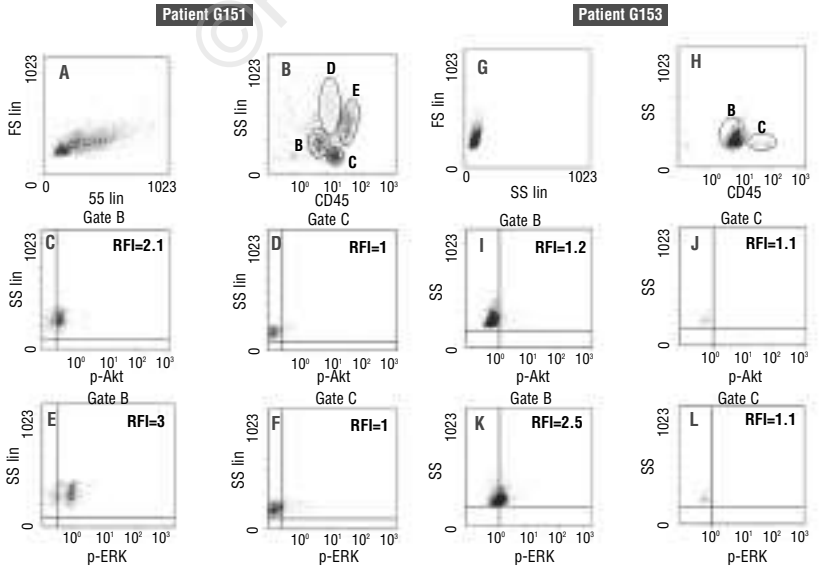

Figure 3 . **Flow cytometric analysis by gating the CD45^{low} blast cell population. (A-F) Flow cytometric analysis of a blood sample from patient G151 using CD45 labeling. (G-L) Corresponding analysis of a bone marrow sample from patient G153. (A and G) FS versus SS dot plot, (B and H) CD45 versus SS dot plot. The blast cell population is CD45^{low} and is detected in gate B, the lymphocytic population is seen in gate C, granulocytic and monocytic populations are detected in gates D and E, respectively, (C and I) p-Akt expression and RFI in gate B. (D and J) p-Akt expression and RFI in gate C. (E and K) p-ERK expression and RFI in gate B. (F and L) p-ERK expression and RFI in gate C.**

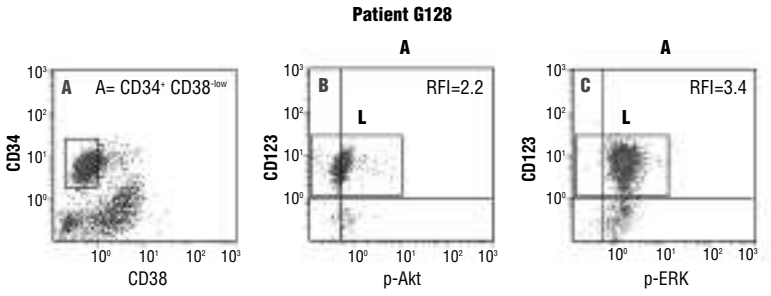

Figure 4. PI3K/Akt and ERK activation in the CD34$^+$ CD38$^{neg/low}$ CD123$^+$ population of immature leukemia cells determined by four-color immunostaining. (A-C) Flow cytometric analysis of a sample from patient G128 using the four-color protocol (A) CD34 versus CD38 dot plot: CD34$^+$ CD38$^{neg/low}$ blast cell population is represented in gate A. (B) CD123 and p-Akt expression in gate A: the CD34$^+$ CD38$^{neg/low}$ CD123$^+$ population is represented in gate L; (C) CD123 and p-ERK expression in gate A. RFI are indicated in the dot plots.

stained with FITC-conjugated anti p-Akt or FITC-conjugated anti p-ERK. Table 2 provides the main characteristics of these patients and the results of the analysis. The percentage of immature cells defined by the CD34$^+$ CD38$^{-/low}$ CD123$^+$ phenotype was highly variable, ranging from 0.1% to 36% of the total cell population (Table 2). In two normal bone marrow samples, we could not detect cells with a CD34$^+$ CD38$^{-/low}$ CD123$^+$ phenotype (*data not shown*). Figure 4 shows the results obtained for patient G128 with AML2. Dot plots of CD34-PC7 versus CD38-PE versus CD123-PC5 allowed identification of the immature population in gate (L). In this patient, these cells represented 36% of the whole cell population and showed Akt (RFI= 2.2) and ERK (RFI= 3.4) phosphorylation. Overall, in this immature leukemic cell population, we detected Akt phosphorylation in three of six AML samples and ERK phosphorylation in all five AML samples tested (Table 2). Interestingly, constitutive activation of these signaling pathways was similar in total CD34$^+$ blasts and in immature leukemic cells. This was also true when the immature population only constituted a very small fraction of the CD34$^+$ blasts (Table 2, see patients G141 and G145). Thus, four-color immunostaining is an efficient method for studying the activation of signaling pathways in immature blast cells and for following their response to novel targeted therapies.

Discussion

AML is an aggressive malignancy and most patients will eventually die from their disease. Advances in understanding the molecular and biological mechanisms leading to AML have provided targets for new therapeutic agents. In particular, abnormal activation of the STAT,[23,24] ERK/MAPK, PI3K/Akt, mTORC1[9,25] and NF-κB[20,26,27] signal transduction pathways has been detected in AML blasts, and may contribute to proliferation, survival and/or self renewal of leukemia stem cells. Specific inhibition of these signaling pathways may constitute a new step in the development of molecularly targeted therapies for AML.

As the development of specific inhibitors targeting the signaling pathways proceeds, identifying the activation status of the corresponding pathways in every patient with AML, whatever the phenotype, the number of blast cells available for analysis, and the percentage of blasts in the bone marrow or blood, becomes essential. Moreover, the need for sensitive analytical methods to monitor the inhibition of target phosphorylation is becoming of great importance. Western blotting is the reference technique for the study of phosphorylated proteins, but it has several drawbacks in AML: (i) it needs at least 1-2×10^6 cells/sample; (ii) it is time-consuming (it requires a minumum of 2 days); (iii) it can only be used for samples with high blast infiltration since the cell population is analyzed as a whole. Consequently, flow cytometry analysis may become the preferred technique. This analysis can be achieved in less than three hours. This short period should be important for monitoring a patient's response to a specific inhibitor, and for determining whether sufficient levels of the inhibitor are reached in the patient's serum to inhibit the targeted pathways in leukemic cells and to adapt therapy accordingly. This follow-up of the inhibition efficiency can be achieved without major drawbacks since our results show that highly reliable results can be obtained from blood samples (see Figure 3). Flow cytometry requires fewer cells: 1×10^5 cells are sufficient. Furthermore, cells can be simultaneously tested for signaling kinase activation and phenotyped, thereby allowing the determination of signaling pathway activation in minor cell populations. Finally, flow cytometry can be performed in most hematology laboratories.

Intracellular phospho-protein staining techniques for flow cytometry allow signaling events to be monitored at the level of the single cell.[28–31] Nolan's group reported that the phospho-protein response panel distinguished leukemic signal transduction networks and measured the phosphorylation state at the level of individual cells in complex populations.[32] Given the availability of phospho-specific antibodies directed against Ser 473 and Thr 308 of Akt kinase, a previous study showed that double staining of CD33 and cytoplasmic p-Akt was possible in AML samples and that cryopreservation did not affect the results of flow cytometry analysis.[33]

In this work, we compared two techniques, western blotting and flow cytometry, which are available for studying the activation of the PI3K/Akt and MAPK signaling pathways. We studied 32 patients with CD34$^+$ AML and highly infiltrated bone marrow. As reported in Table 1, the agreement between the two techniques was 100% for both Akt and ERK activation. We then studied the activation of these two signaling pathways in 20 additional samples from patients with either a low percentage of bone marrow blast infiltration or a small number of cells insufficient for western blot analysis.

The percentage of patients with constitutive ERK or Akt activation in this series was the same as that in our first series of patients, showing that this method is also reliable in samples from patients with low bone marrow infiltration. Altogether, the percentage of constitutive Akt activation was 46% and that of ERK activation was 68%; a similar percentage of spontaneous ERK activation (74%) was previously reported[16] whereas higher levels of Akt constitutive activation in AML were reported by two groups.[8,9] We found three different signaling profiles in our patient series: patients without any activation, patients with ERK activation only and a third group of patients with activation of both pathways. The reason for these differences is currently not understood and does not correlate with cytogenetics (Table 1). The prognostic value of abnormal activation of Akt and/or ERK will be evaluated as soon as the duration of the patients' follow-up is sufficient.

Thirty-nine percent of the AML patients studied in our department were CD34 negative, which is within the previously reported range of CD34 expression in AML.[34,35] In order to study all patients with AML at diagnosis for the PI3K/Akt and ERK/MAPK pathways, we designed a flow cytometry assay using double staining with CD45 and either anti-p-Akt or anti-p-ERK antibodies. By gating on $CD45^{low}$ expression and identifying blast cells in gate B (Figure 3B), we were able to determine the status of activation of these two signaling pathways. We suggest that this technique could become the usual technique for studying signaling pathway activation in AML blasts. Indeed, this technique is rapid, since it can be performed in 3 hours. Moreover, directly conjugated antibodies are now commercially available, thereby making this technique easier and faster. This flow cytometry method will be of interest for assessing the response to new targeted treatments, as recently described in chronic myeloid leukemia.[29]

Finally, our work was aimed at studying the activation of the PI3K/Akt and Ras/ERK pathways in immature leukemia cells including the minority subset of leukemic stem cells. These cells are of critical importance in the biology of AML. They have long-term self-renewal capacity and are responsible for the production of clonogenic leukemic cells. It is likely that current chemotherapeutic agents only target clonogenic tumor cells but spare the leukemic stem cells. Identifying abnormal activation of signaling pathways in these leukemic stem cells, as compared to normal hematopoietic stem cells, is a great challenge for the future development of targeted therapies.[20,26] The leukemic stem cells belong to a cell population whose immunophenotype has been determined to be $CD34^{+}$, $CD38^{-/low}$, $CD90^{-}$, $CD123^{+}$,[19] $CD71^{-}$, $CD117^{-}$, $HLADR^{-}$.[36] To date, no study has reported an antigenic difference between leukemic stem cells derived from distinct AML subtypes. We used four-color flow cytometry in order to study p-Akt and p-ERK phosphorylation in cells defined by the $CD34^{+}$, $CD38^{-/low}$, $CD123^{+}$ phenotype. This population of AML blast cells is known to contain the SCID-leukemia initiating cells[37] and thus, constitutes the specific target for new therapies to be developed. We did not detect CD123 expression on the $CD34^{+}$, $CD38^{-/low}$ population in normal bone marrow controls and it has been reported that the expression of CD123 is significantly higher in AML than in normal bone marrow.[21] In the immature $CD34^{+}$ $CD38^{-/low}$ $CD123^{+}$ population, we detected Akt phosphorylation in three out of six patients and ERK1/2 activation in all five patients analyzed. Our results demonstrate that these pathways are already activated in immature leukemic cells, thus confirming that these signaling pathways are potential interesting targets for new therapies for AML.

VB and JT performed the experiments and analyzed the data; NI and FD provided bone marrow samples and essential clinical information on patients; PM participated in the conception of the study; DB and CL conceived and directed the study. We are indebted to Professor M.C. Bene (CHU Nancy) for helpful criticism and comments and to Professor P. Anract (H. Cochin) for providing normal bone marrow samples. The authors declare that they have no conflicts of interest.

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