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Flow Cytometric Detection of Total and Serine 473 Phosphorylated Akt

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Abstract The evaluation of regulatory proteins is important for biological studies and is also established as a prognostic marker for cancer diagnosis. Very recently, it has been highlighted that the serine/threonine kinase Akt plays a fundamental role in survival pathways and is also involved in the onset of resistance to anti-neoplastic drugs and ionizing radiation in cell lines derived from solid tumors. For its full activation Akt needs to be phosphorylated on Serine 473 residue. Molecules that are fundamental in determining resistance to therapeutic treatments might serve in the future as clinical markers to tailor therapy and/or predict treatment response. The aim of this study was to ascertain whether or not flow cytometric analysis of total Akt and of its form phosphorylated on Serine 473 could be related to standard techniques such as Western blotting with phosphospecific antibodies and in vitro kinase assay. To this end, we employed as experimental models HL-60 and PC-12 lines in which there is an enhancement of Akt activity. Our results showed that flow cytometry analysis, performed on fixed and permeabilized cells, correlated well with the results provided by in vitro activity assays and Western blots. Therefore, our findings might indicate that flow cytometric study of Akt (both total and phosphorylated) content may be applied in routine work for phenotyping of hematological and non-hematological neoplasias, and allow for its use as a useful marker for the classification and the prognosis of neoplastic diseases. J. Cell. Biochem. 86: 704–715, 2002. © 2002 Wiley-Liss, Inc.

Key words: Akt; phosphorylation; flow cytometry; Western blot; activity assay

The serine/threonine protein kinase Akt (also know as PKB, for protein kinase B) is the cellular homologue of the v-akt oncogene [Brazil and Hemmings, 2001; Testa and Bellacosa, 2001]. To date, three different Akt isoforms have been identified: Akt-1, -2, and -3, that are the products of different genes, even though they are highly related and exhibit greater than 80% homology at the amino acid level [Nicholson and Anderson, 2002]. Each Akt isoform possesses an N-terminal pleckstrin homology (PH) domain of approximately 100 amino acids. The PH domain mediates binding of Akt to 3-phosphoinositides that are the products of phosphoinositide 3-kinases (PI3Ks). The PH domain is followed by the kinase catalytic domain, which displays a threonine residue (Thr 308 in Akt1) whose phosphorylation is necessary for Akt activation. Following the kinase domain is a hydrophobic C-terminal tail containing a second regulatory phosphorylation site in a serine residue (Ser 473 in Akt1) [Chan et al., 1999]. Akt is activated in response to many stimuli including growth

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factors such as insulin, insulin-like growth factor-I, epidermal growth factor, or cytokines such as interleukin-3, interleukin-6, macrophage-colony stimulating factor [Datta et al., 1999; Lawlor and Alessi, 2001]. These agonists stimulate PI3K which synthesizes 3-phosphoinositides such as phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) [Vanhaesebroeck and Waterfield, 1999; Cantrell, 2001]. PtdIns(3,4,5)P_3 activates the phosphoinositide-dependent protein kinase PDK-1 which is recruited at the plasma membrane [Vanhaesebroeck and Alessi, 2000; Vanhaesebroeck et al., 2001]. PDK-1 phosphorylates Akt on Thr 308. It is commonly thought that PtdIns (3,4,5)P_3 causes a conformational change in Akt with an ensuing increase of the accessibility to the phosphorylation site. However, PtdIns (3,4,5)P_3 also induces colocalization of Akt with PDK-1 at the plasma membrane. For a full activation of Akt, phosphorylation of Ser 473 is also required. The kinase responsible for this phosphorylation is yet to be identified, but it might be PDK-1 or PDK-2, ILK (integrin-linked kinase) or Akt itself [Brazil and Hemmings, 2001; Nicholson and Anderson, 2002]. Once activated, Akt phosphorylates several downstream targets [Nicholson and Anderson, 2002]. Evidence indicates that Akt plays a key role in cancer progression by stimulating cell proliferation and inhibiting apoptosis [Lawlor and Alessi, 2001; Testa and Bellacosa, 2001]. Recently, a few reports highlighted that Akt is constitutively phosphorylated and promotes cell survival and both drug and ionizing radiation resistance in some cell lines derived from lung or pancreas tumors [e.g., Asselin et al., 2001; Brognard et al., 2001]. For this reason, it has been proposed that evaluation of Akt phosphorylation status might gain in the future a prognostic relevance in these diseases [Brognard et al., 2001]. Detection of Akt phosphorylation relies on the use of antibodies specific for Thr 308 or Ser 473 phosphorylated Akt (p-Akt) and Western blotting analysis [Lawlor and Alessi, 2001]. However, Western blots are time-consuming to perform and require substantial amounts of cells. This can be a limiting factor if pathological specimens are to be analyzed. Conversely, flow cytometry is a technique which is more rapid to perform and requires lower numbers of cells in comparison with Western blot. It also makes it possible to evaluate single cells. This latter characteristic allows for the possibility to define cell subsets, and, eventually, to perform cell sorting of pre-defined cell populations. Moreover, flow cytometric analysis of cells stained for cytoplasmic antigens is now well standardized and routinely performed [Curti et al., 2001, 2002]. In particular, oncogene products and cell cycle-related proteins can be analyzed and quantified [Bradbury et al., 1997; Keng and Siemann, 1998; Milosevic, 2000], and different approaches (e.g., analysis of phosphorylated compounds, double staining with surface markers and distribution of the antigen along the cell-cycle by contemporary staining with propidium iodide) might be devised.

In this article, we demonstrate that flow cytometry can be effectively coupled to Akt antibodies to detect both total Akt and Ser 473 p-Akt, thus making it possible a comparison between enzymatic activity, Western blot and flow cytometric analysis.

MATERIALS AND METHODS

Materials

RPMI 1640, D-MEM, fetal calf serum, normal rabbit IgG, normal mouse IgG, normal goat serum (NGS), monoclonal antibody to β-tubulin, protein A-Agarose, affinity-purified rabbit or mouse IgG, peroxidase-conjugated anti-mouse or anti-rabbit IgG and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Nerve growth factor (NGF) was from Upstate Biotechnology (Lake Placid, NY). Histone H2B and the Lumi-Light^{Plus}enhanced chemiluminescence (ECL) detection kit were from Roche Molecular Biochemicals (Milan, Italy). [γ-^{32}P] ATP was from Amersham Pharmacia Biotech (Uppsala, Sweden). Ly 294002 and wortmannin were from Calbiochem, La Jolla, CA. The Protein Assay kit (detergent compatible) was from Bio-Rad (Hercules, CA). cAMP-dependent protein kinase inhibitor peptide was from Bachem (Bubendorf, Switzerland). P-81 paper was from Whatman (Maidstone, UK).

The following antibodies were obtained from Cell Signaling Technology, Beverly, MA: to total Akt (Catalog #2966, mouse monoclonal, and Catalog #9272, rabbit polyclonal), to Ser 473 p-Akt (Catalog #9271, rabbit polyclonal, specific for Western blot), to Ser 473 p-Akt (Catalog #9277, rabbit polyclonal, specific for immunohistochemistry). Fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (for

flow cytometry) was obtained from DAKO, Copenhagen, Denmark. FITC-conjugated goat anti-mouse IgG (Fc fragment) and the kit for cytoplasmic staining (Intraprep) were obtained from Coulter-Immunotech, Miami, FL.

Cell Culture

The HL-60 parental cell line (HL-60PT), obtained from the American Type Culture Collection (CCL 240), was routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum at an optimal cell density of $3–8 \times 10^5$ cells/ml. To down-regulate Akt activity, cells were treated for 24 h with either wortmannin (100 nM) or Ly294002 (5 μM). Rat pheocromocytoma PC-12 cells were cultured in D-MEM containing 10% heat-inactivated horse serum and 5% heat-inactivated fetal calf serum. Prior to stimulation with 100 ng/ml NGF for the indicated times, cells were switched to D-MEM containing 1% horse serum for 48 h. In some cases, cells were pre-treated for 1 h prior to NGF exposure with either wortmannin (100 nM) or Ly294002 (50 μM). Prior to further analyses, PC-12 cells were detached from culture plates by means of cold PBS containing 0.5 mM EDTA.

Isolation of a Variant HL-60 Cell Clone

HL-60 cells resistant to camptothecin [Martelli et al., 1999a] were diluted at a density of about 100 cells for 10 ml of filtered, conditioned RPMI medium. Before plating in tissue culture plates, the cell suspension was maintained at 37°C and supplemented with sterile agarose at the final concentration of 0.18%. After 2 weeks, clones were picked with a Pasteur pipette, and expanded in complete medium.

Preparations of Cell Homogenates for Western Blot Analysis

Cells were sedimented at 1,000 g for 10 min and washed twice in PBS containing the COMPLETE Protease Inhibitor Cocktail (Roche Molecular Biochemicals), according to the manufacturer's instructions, supplemented with 1.0 mM Na_3VO_4 and 20 nM okadaic acid. Cells were then resuspended at $\approx 10^7$/ml in boiling lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecylsulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and the protease and phosphatase inhibitor cocktail. Lysates were briefly sonicated to shear DNA and reduce viscosity, boiled for 5 min to solubilize protein, and stored at −80°C until required.

Western Blot Analysis

Protein (50–80 μg) separated on SDS-polyacrylamide gels was transferred to nitrocellulose sheets using a semi-dry blotting apparatus. Sheets were saturated in PBS containing 5% NGS and 4% BSA for 60 min at 37°C (blocking buffer), then incubated overnight at 4°C in blocking buffer containing the primary antibodies. After four washes in PBS containing 0.1% Tween-20, they were incubated for 30 min at room temperature with the appropriate peroxidase-conjugated secondary antibodies, diluted 1:5,000 in PBS-Tween-20, and washed as above. Bands were visualized by the ECL method. To ensure equal loading, blots were always first probed with an antibody to β-tubulin, then stripped and re-probed.

Akt Immunoprecipitation

To obtain homogenates, cells were resuspended in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, supplemented with the protease and phosphatase inhibitor cocktail and homogenized by 30 passages through a 25 gauge needle. Cell homogenates (1 ml, containing 500 μg of protein) were pre-cleared by adding 5 μg of normal rabbit IgG and 10 μg of 50% Protein A-Agarose, followed by incubation for 1 h at 4°C and centrifugation at 12,000*g* for 10 min at 4°C. Then, the samples were incubated for 2 h at 4°C under constant agitation with 5 μg of a polyclonal antibody to total Akt (9272). Ten microgram of 50% Protein A-Agarose was then added and incubation proceeded for 1 h at 4°C under constant agitation.

Akt Activity Assay

The immunoprecipitates were washed twice in lysis buffer, once in distilled water and twice in the Akt kinase buffer (20 mM HEPES-NaOH, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT) as previously reported [Borgatti et al., 2000]. Assays (100 μl) contained 20 mM HEPES-NaOH, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 1 μM cAMP-dependent protein kinase inhibitor peptide, 5 μg histone H2B as exogenous substrate, 2 μM ATP, 10 μCi [γ-^{32}P] ATP (3,000 Ci/mmole). Samples were incubated for 30 min at 30°C and the reaction was then stopped by spotting 80 μl on to P-81 filter papers and immersing in 1% (v/v) orthophosphoric acid. The papers were washed several times,

rinsed in ethanol, air-dried, and the radioactivity was determined by scintillation counting. Background values, obtained by samples in which the antibody to total Akt was replaced by normal rabbit IgG, were subtracted from all values.

Intracellular Immunostaining and Flow Cytometric Analysis

Cells, cultured as described, were collected and centrifuged (600*g*) for 5 min and washed twice with PBS, pH 7.2, and fixed with Reagent 1 of the Intraprep kit, following manufacturer's instructions. Cells were then washed twice and permeabilized with saponin-based Reagent 2 and incubated at 4°C for 12 h with a 1:10 final dilution of antibody to Ser 473 p-Akt (9277, specific for immunohistochemistry, as specified in the manufacturer's leaflet). After this incubation, samples were washed twice with PBS and pellets were incubated with 5 μl of FITC-conjugated swine anti-rabbit IgG. Negative controls were run with FITC-conjugated swine anti-rabbit IgG alone and with FITC-conjugated swine anti-rabbit IgG plus normal rabbit IgG.

In an additional series of experiments, we also utilized a mouse monoclonal antibody against total Akt (2966) which was revealed with a FITC-conjugated anti-mouse IgG, as well as the polyclonal antibody to Ser 473 p-Akt specific for Western blot (9271). Negative targets were lymphocytes and granulocytes obtained from normal donors, after informed consent, as required by the Ethical Committee of the S.Orsola-Malpighi Hospital. The latter cells were incubated with anti Ser 473 p-Akt (specific for immunocytochemistry) as described above.

All the samples were analyzed by EPICS XL and EPICS ELITE flow cytometers (Coulter, Hialeah, FL) equipped with dedicated softwares. Histograms were then analyzed with EXPO software.

The histograms showed are representative of three separate experiments.

RESULTS

Akt Activity in an Apoptosis Resistant Clone of HL-60 Cells

In the course of our previous studies on apoptosis [Martelli et al., 1999a,b,c], we fortuitously selected a cell population which was resistant to drugs, such as camptothecin and etoposide, that we employed to induce apoptosis in HL-60 cells. A clone was further isolated on soft agar. We named this clone HL-60AR cells (for apoptosis resistant). A detailed study on the characterization of this clone will be published elsewhere. As shown in Figure 1A, Western blotting analysis revealed that the amount of total Akt was similar in HL-60PT and AR cells. However, when the blots were probed with an antibody to Ser 473 p-Akt, it became evident that HL-60AR cells contained more than HL-60PT cells (Fig. 1B). The levels of Ser 473 p-Akt in AR cells could be markedly reduced by a 24-h incubation in the presence of two pharmacological inhibitors of the catalytic subunit of PI3K, i.e., wortmannin and Ly294002. Consistently, the in vitro kinase activity of Akt was higher in HL-60AR when compared to PT cells. The activity could be markedly inhibited by treatment with either wortmannin or Ly294002 (Fig. 1C).

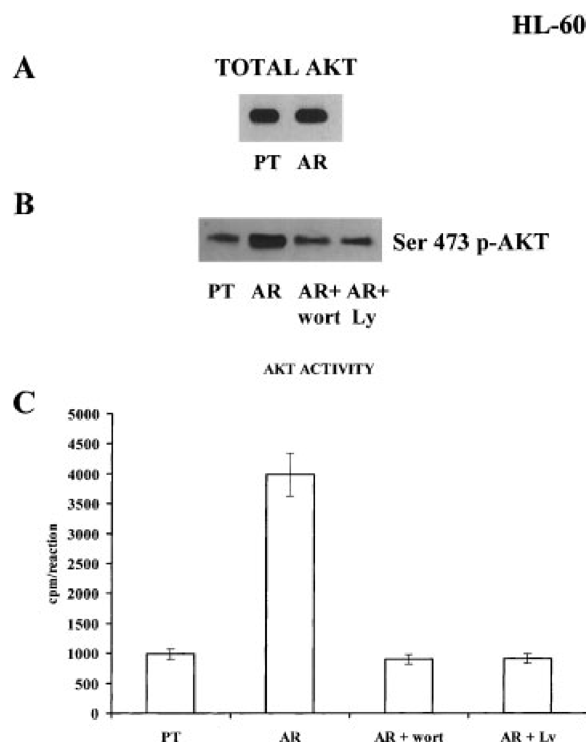

Fig. 1. Akt activity in HL-60 cells. (**A**) Western blotting analysis of total Akt in HL-60PT and AR cells; antibody 2966 was employed. (**B**) Western blotting analysis of Ser 473 p-Akt in HL-60PT and AR cells; antibody 9271 was utilized. (**C**) Assay of in vitro Akt kinase activity on histone H2B in HL-60PT and AR cells; for the immunoprecipitation antibody 9272 was used. In (A) and (B), 60 μg of protein was blotted to each lane. In (C), the results are the mean ± SD of three separate experiments. Wort, wortmannin; Ly, Ly294002.

Akt Activity in NGF-Treated PC-12 Cells

We next investigated Akt in PC-12 rat pheochromocytoma cells treated with NGF. As presented in Figure 2A, the amount of total Akt did not change following a 15 min incubation in the presence of NGF, irrespectively of the presence of PI3K inhibitors, wortmannin and Ly294002. However, in response to NGF, there was a marked increase in the amount of Ser 473 p-Akt, which could be prevented by the use of PI3K inhibitors (Fig. 2B). Results provided by Western blotting were corroborated by in vitro Akt kinase assays that showed an increase in Akt activity elicited by NGF treatment. The increase was blocked by pre-incubation with either wortmannin or Ly294002 (Fig. 2C).

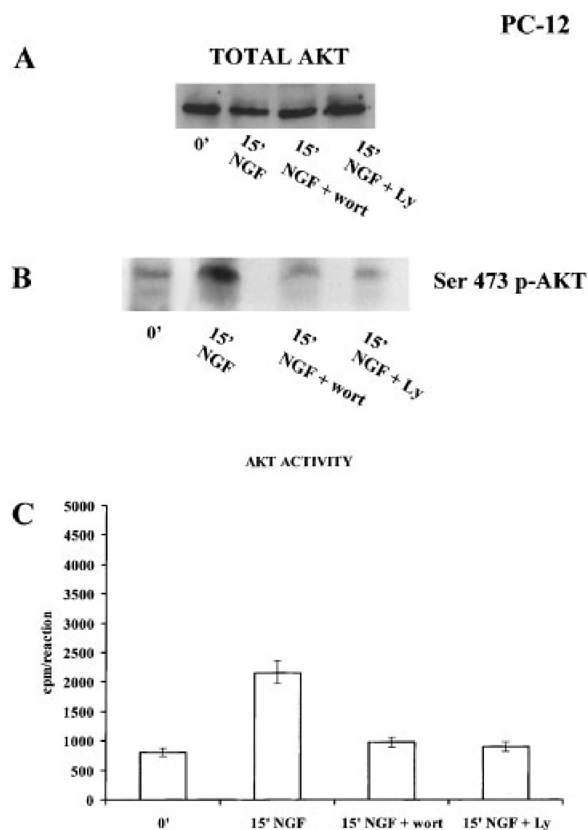

Fig. 2. Akt activity in PC-12 cells. (**A**) Western blotting analysis of total Akt in control (0′) and NGF-treated cells; antibody 2966 was employed. (**B**) Western blotting analysis of Ser 473 p-Akt in control (0′) and NGF-treated cells; antibody 9271 was utilized. (**C**) assay of in vitro Akt kinase activity on histone H2B in control (0′) and NGF-treated cells; for the immunoprecipitation antibody 9272 was used. In (A) and (B) 80 μg of protein was blotted to each lane. In (C), the results are the mean ± SD of three separate experiments. Wort, wortmannin; Ly, Ly294002.

Flow Cytometric Analysis of Akt

We investigated the possibility of flow cytometric analysis of the content of total Akt and Ser 473 p-Akt, and whether or not the obtained results could be related to both enzymatic activity and Western blot analysis. First, we performed a standard cytoplasmic staining protocol for HL-60PT and AR cells using the antibody to Ser 473 p-Akt specific for immunocytochemistry. We found that, in comparison to controls, p-Akt staining was detectable in HL-60AR cells, whereas PT cells displayed a small subset of weakly stained cells (see Fig. 3 for a typical example of staining). Moreover, HL-60AR cells were positive for p-Akt with a shift to the right of the histogram of at least 1 decade (1 Log) of intensity, measured as mean channel value of fluorescence (MCV). The results were always easily detected, and morphology was well maintained in fixed and permeabilized cells. In addition, experiments were performed to lower the content of Ser 473 p-Akt, by incubating HL-60AR cells with wortmannin or Ly294002, as described in the Materials and Methods. According to Western blot analysis, content of p-Akt was diminished in treated HL-60AR cells (see Fig. 1B). As presented in Figure 4, flow cytometric analysis provided results showing a shift to the right which is weaker in comparison to untreated AR cells, being intermediate between the negative control and the untreated HL-60AR cells. Thus, the effects of either wortmannin or Ly294002 were readily detectable by flow cytometry in a quicker fashion, in comparison to the time required by activity assays or Western blot, but with similar results. We also tested the positivity for total Akt in HL-60 cells, both PT and AR, by means of a monoclonal antibody. As expected from Western blot results, total Akt was well recognized in both the cell types, with no obvious differences (Fig. 5). Moreover, given that the antibody to Ser 473 p-Akt we employed for Western blots was different from that used for cytometric analysis, we sought to determine if also the former was suitable for flow cytometry. As presented in Figure 5, also this antibody detected a marked increase in the amount of Ser 473 p-Akt in HL-60AR when compared with PT cells.

In a further set of experiments, we applied the same technique to a well-known model of p-Akt induction, employing NGF-stimulated PC-12 cells. In fact, Ser 473 p-Akt is quickly inducible

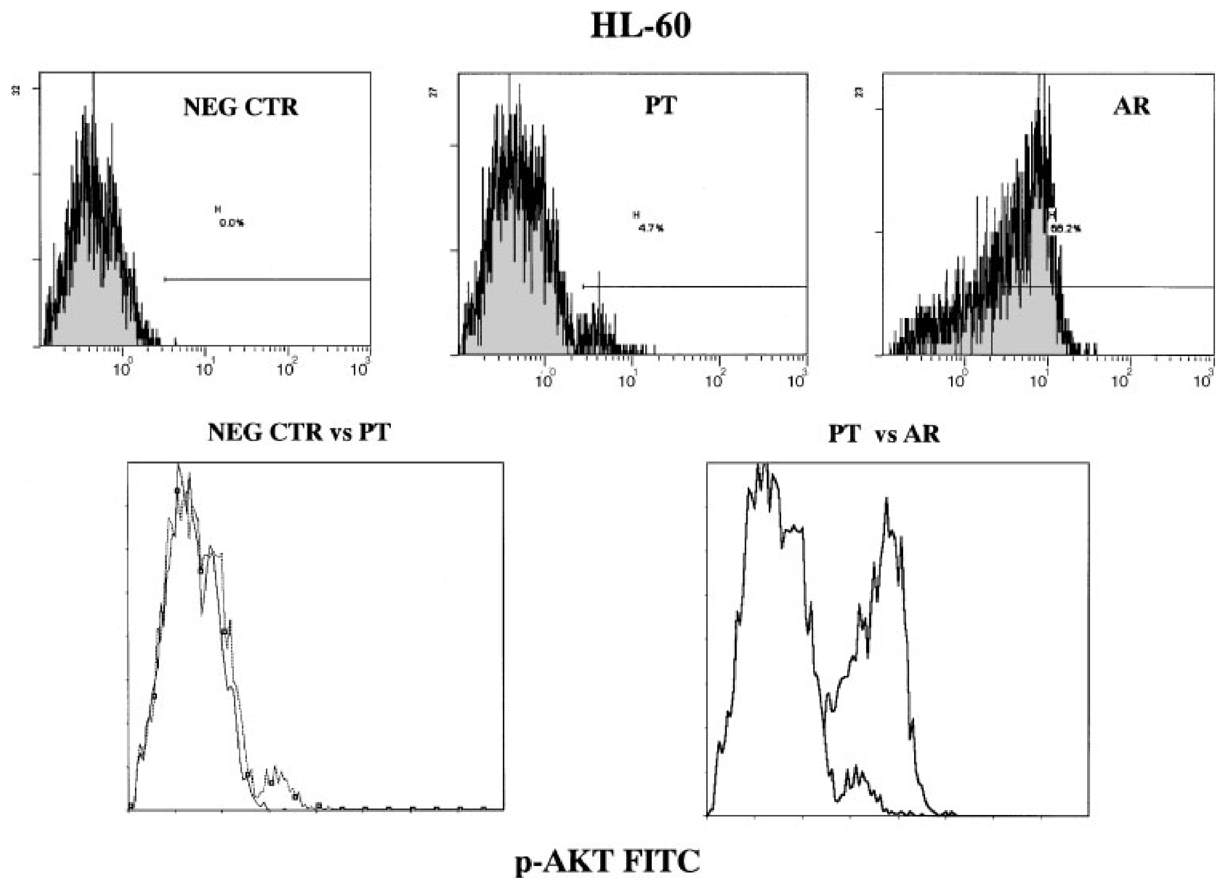

Fig. 3. Flow cytometric analysis of Ser 473 p-Akt in HL-60 cells. The histograms show the content of p-Akt, as evaluated by flow cytometry, in HL-60PT and AR cells, utilizing the polyclonal antibody specific for immunocytochemistry (9277). Note how a small subset positive for p-Akt is present in HL-60PT cells. Negative controls (neg ctr) were run with FITC-conjugated swine anti-rabbit IgG plus normal rabbit IgG.

by NGF treatment within 15 min (see Fig. 2B). As shown in Figure 6, positivity for p-Akt (antibody specific for immunocytochemistry) was detected in NGF-treated cells with a MCV shift of more than 1 Log (1 decade) in comparison with serum-starved cells. In analogy with the results of the experiments performed with HL-60AR cells, samples pre-treated with wortmannin or Ly294002 showed a weaker positivity for p-Akt (Fig. 7), when matched with NGF alone-treated cells. Also in this case, we detected an increase of Ser 473 p-Akt in NGF-treated samples when we employed the antibody specific for Western blotting analysis (Fig. 8).

The specificity of the staining was further confirmed utilizing negative targets, i.e., granulocytes and lymphocytes obtained from normal donors (5 subjects). As expected, these cells, stained following the protocol described above, were negative for p-Akt (Fig. 9).

DISCUSSION

Recent evidence indicates that p-Akt might become an important marker for prognosis in human neoplasias as well as a target for therapy, because it stimulates cell proliferation and inhibits apoptosis [Page et al., 2000; Huang and Oliff, 2001]. Thus, there could be the need to devise tests for a rapid evaluation of the presence of this kinase, its degree of phosphorylation and the response to selective pharmacological inhibitors. Akt is usually studied by activity assays or Western blot, that are time consuming techniques and cannot distinguish cell subsets, which are usually present in human cancers. Therefore, flow cytometric analysis might be the best choice for the above purposes, provided that appropriate reagents be available. The aim of this investigation was to ascertain whether or not commercially available antibodies to total Akt and its form

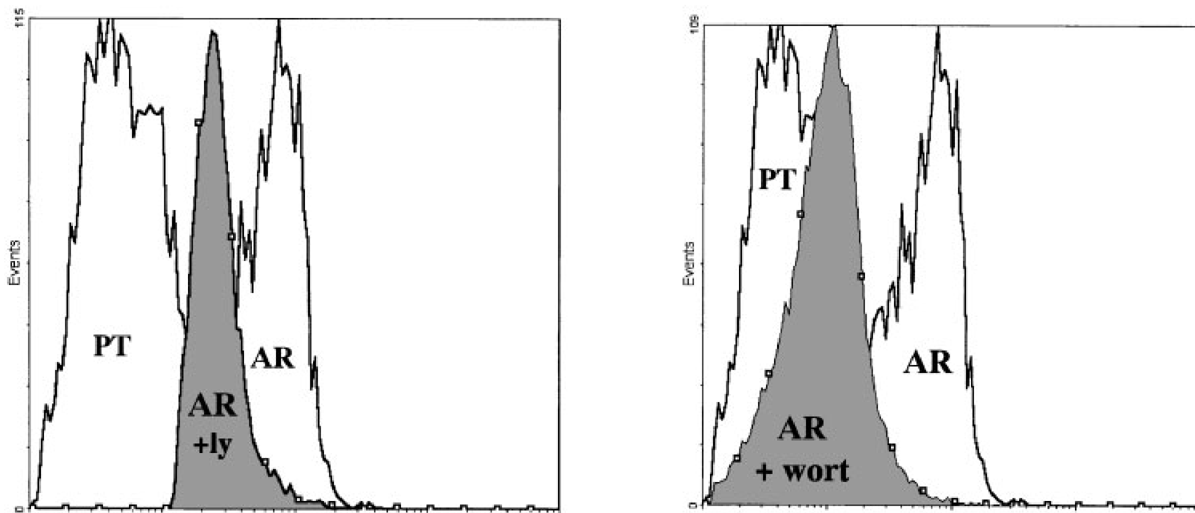

Fig. 4. Inhibitors of PI3K lower Ser 473 p-Akt content in HL-60AR cells. Overlay histograms demonstrate the lower content of p-Akt in cells exposed to either wortmannin (wort) or Ly294002 (ly). The antibody to Ser 473 p-Akt specific for immunocytochemistry was employed (9277).

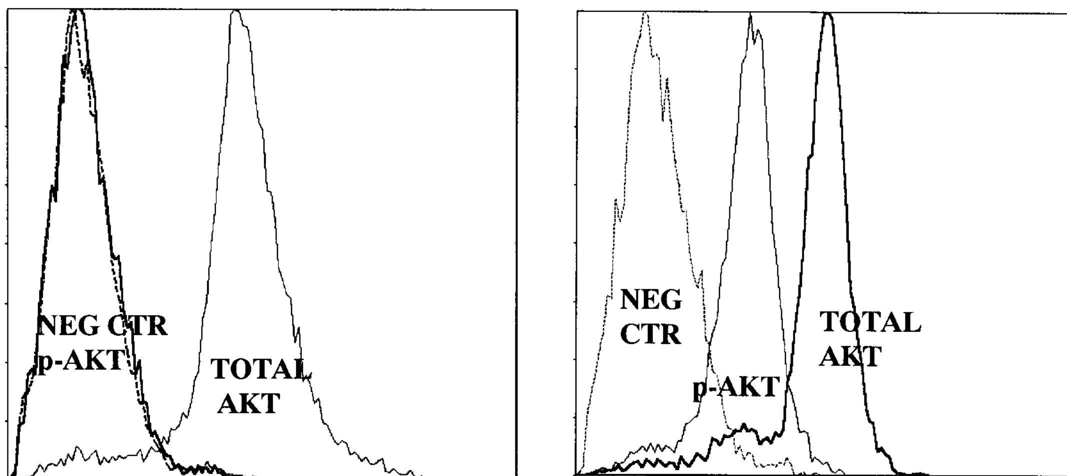

Fig. 5. Flow cytometric analysis of total Akt or Ser 473 p-Akt in HL-60PT and AR cells. Histograms show that the antibodies employed for Western blot detection of total Akt (2966) and Ser 473 p-Akt (9271) recognize their antigens also when utilized for flow cytometric analysis. Negative controls (neg ctr) were run with FITC-conjugated swine anti-rabbit IgG plus normal rabbit IgG for Ser 473 p-Akt and with FITC-conjugated goat anti-mouse IgG plus normal mouse IgG for the total Akt mouse monoclonal antibody.

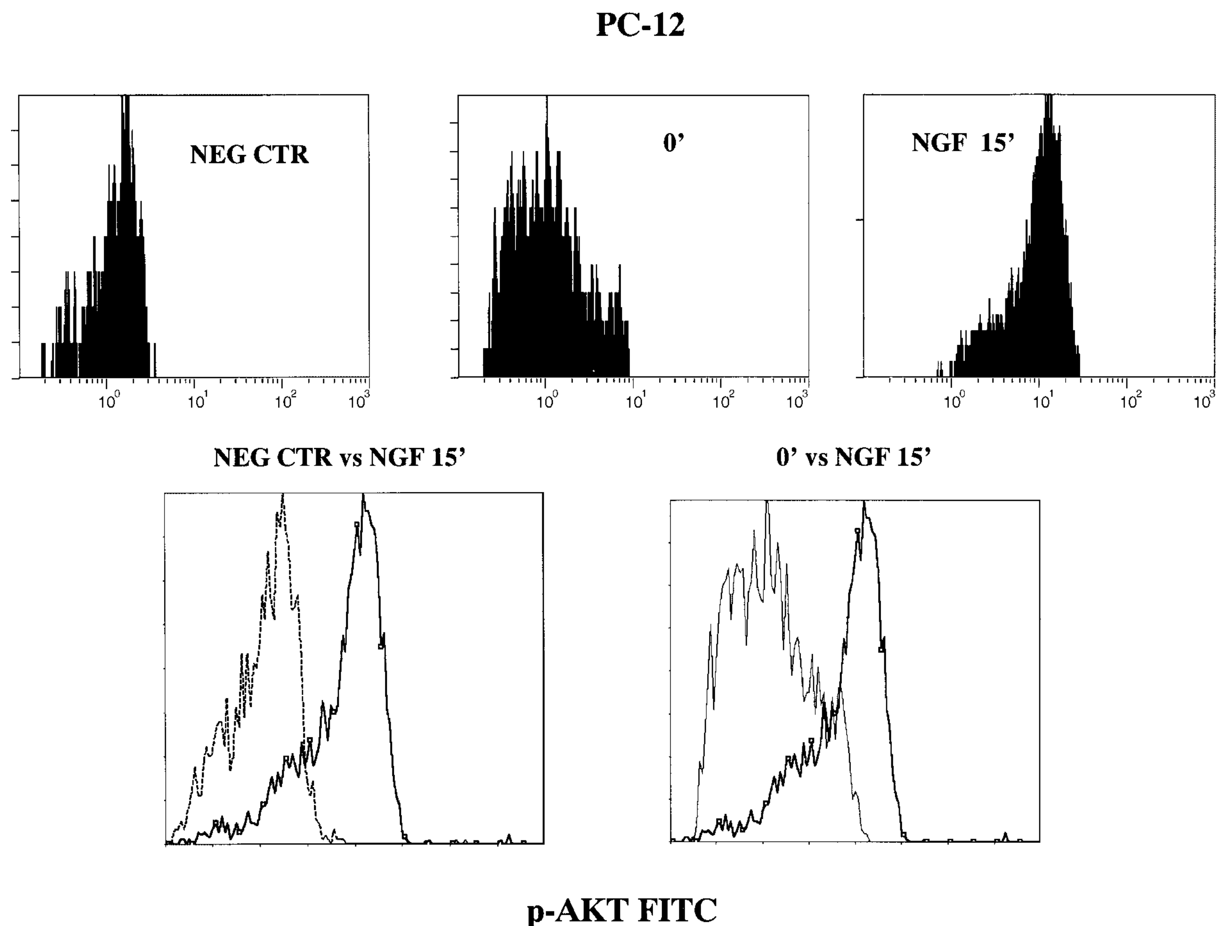

Fig. 6. Flow cytometric analysis of Ser 473 p-Akt in unstimulated (0′) and NGF-treated (15′) PC-12 cells. The histograms show the content of p-Akt, as evaluated by flow cytometry, utilizing the polyclonal antibody specific for immunocytochemistry (9277). Negative controls (neg ctr) were run with FITC-conjugated swine anti-rabbit IgG plus normal rabbit IgG.

phosphorylated on Ser 473 may be employed for a flow cytometric detection. We utilized two experimental models in which there is an activation of Akt. The first one consists of HL-60AR cells, a clone which we have isolated during our own previous studies on apoptosis [Martelli et al., 1999a,b,c]. This clone, when compared with HL-60PT cells, exhibits a constitutive activation of the PI3K/Akt signal transduction pathway. The second one is constituted of PC-12 cells stimulated with NGF [Andjelković et al., 1998]. Our results showed that in both of these experimental models in vitro Akt kinase activity on histone H2B correlated well with the phosphorylation levels of Ser 473 p-Akt revealed by phosphospecific antibodies. On the other hand, Western blot analysis showed that the expression of total Akt was the same in HL-60AR when compared with PT cells and that it did also not change following stimulation of PC-12 cells with NGF. Both Akt kinase activity and phosphorylation level of Ser 473 p-Akt were highly sensitive to two unrelated pharmacological inhibitors of PI3K, wortmannin and Ly294002.

For flow cytometric analysis, we tested the two same antibodies we utilized for Western blotting (one to total Akt and the other to Ser 473 p-Akt). In addition, we used an antibody to Ser 473 p-Akt specific for immunocytochemistry. Indeed, we reasoned that this antibody could be more suitable for flow cytometry because it recognizes p-Akt after formaldehyde fixation, i.e., a step included in our staining protocol for flow cytometric detection.

Since Akt and its phosphorylated forms are intracellular proteins, we utilized a saponin-based method of permeabilization, in analogy with methods aimed at intracellular analysis by flow cytometry of interleukins, bcl-2, and the

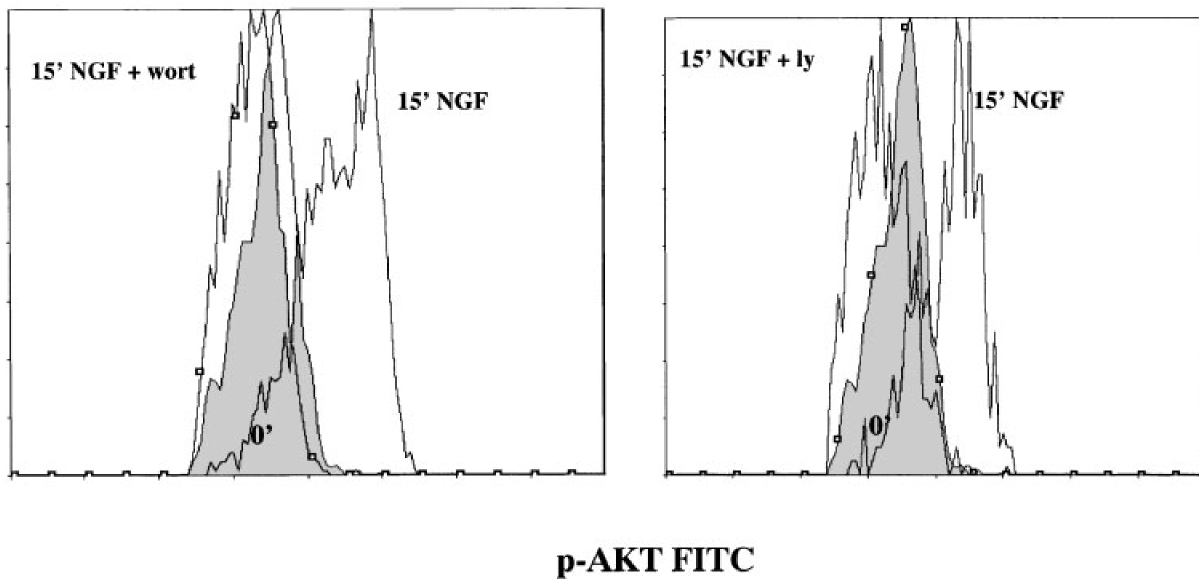

Fig. 7. Inhibitors of PI3K lower Ser 473 p-Akt content in NGF-treated PC-12 cells. Overlay histograms demonstrate the lower content of p-Akt in cells exposed to either wortmannin (wort) or Ly294002 (ly). The antibody to Ser 473 p-Akt specific for immunocytochemistry was employed (9277).

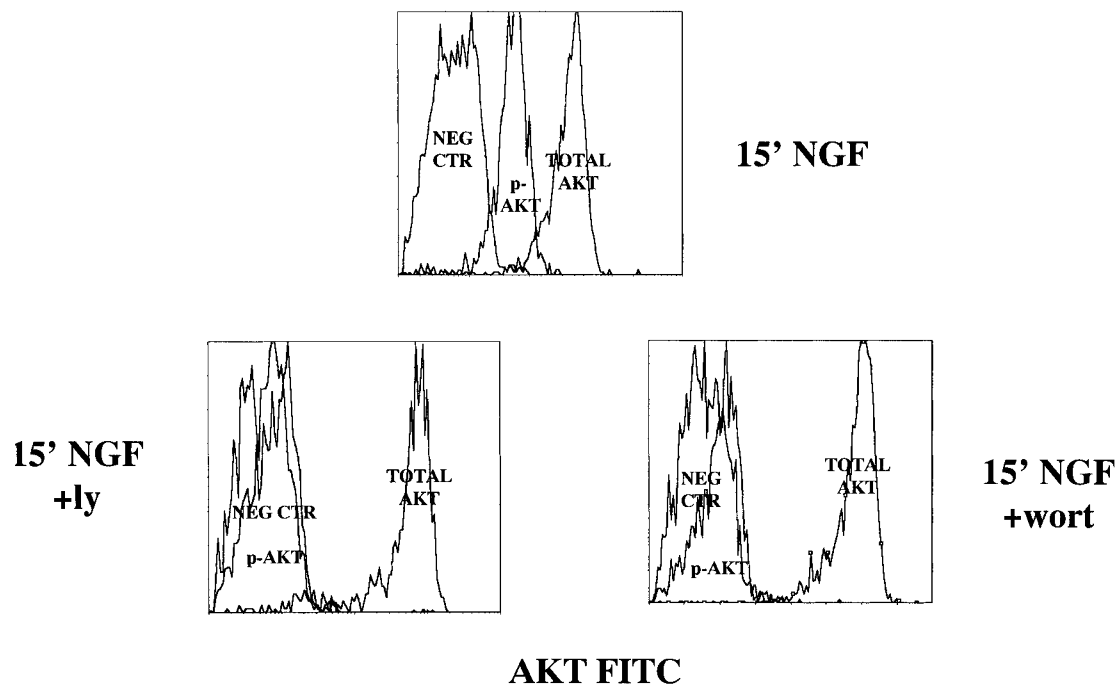

Fig. 8. Flow cytometric analysis of total Akt or Ser 473 p-Akt in NGF-treated PC-12 cells. Histograms show that the antibodies employed for Western blot detection of total Akt (2966) and Ser 473 p-Akt (9271) recognize their antigens also when utilized for flow cytometric analysis. Negative controls (neg ctr) were run with FITC-conjugated swine anti-rabbit IgG plus normal rabbit IgG for the Ser 473 p-Akt rabbit polyclonal antibodies and with FITC-conjugated goat anti-mouse IgG plus normal mouse IgG for the total Akt mouse monoclonal antibody.

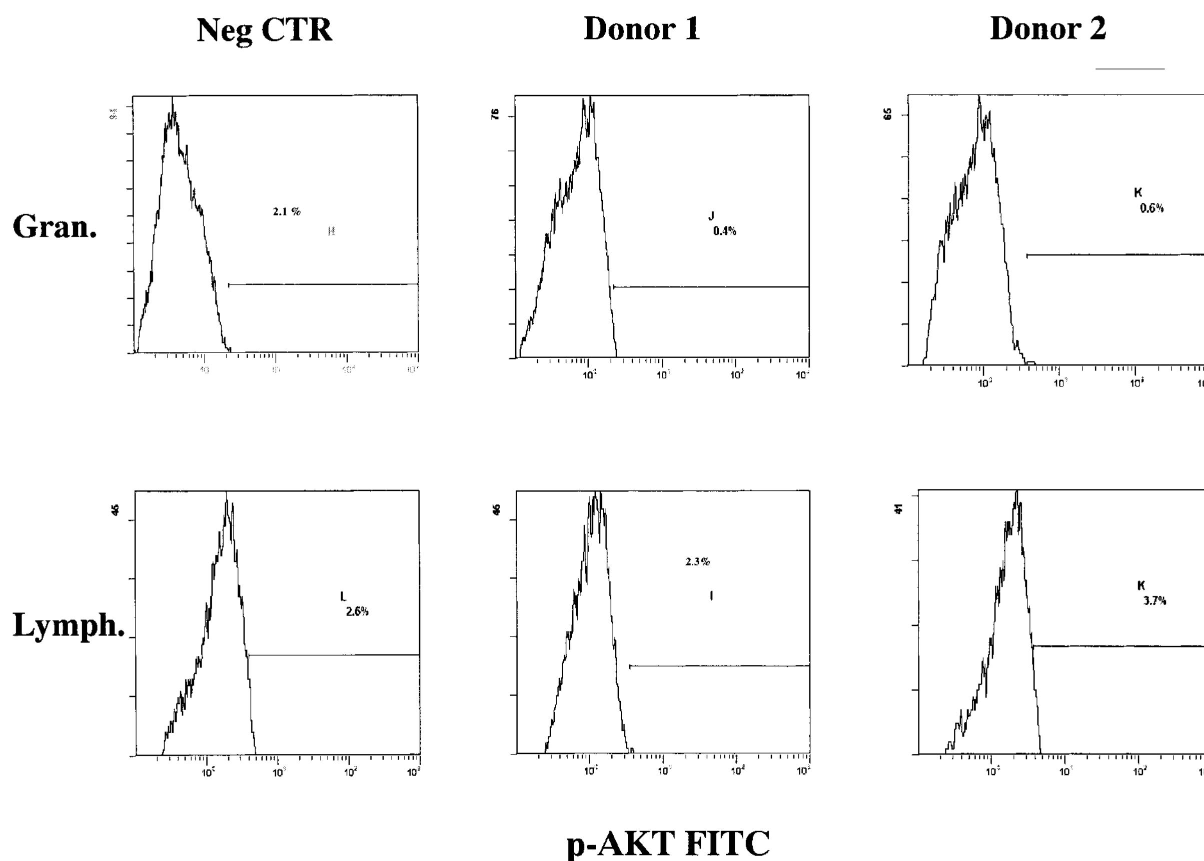

Fig. 9. Granulocytes (Gran.) and lymphocytes (Lymph.) obtained from normal donors are negative when analyzed for p-Akt content by flow cytometry. The antibody to Ser 473 p-Akt specific for immunocytochemistry was employed (9277). Negative controls (neg ctr) were run with FITC-conjugated swine anti-rabbit IgG plus normal rabbit IgG.

proliferation-related antigen Ki-67 [Bradbury et al., 1997; Keng and Siemann, 1998; Curti et al., 2001, 2002]. The results obtained by means of flow cytometric analysis were in good agreement with the findings provided by in vitro Akt kinase assay or Western blotting. The levels of total Akt were similar in HL-60PT and AR cells or in PC-12 cells prior and after stimulation with NGF. Regarding changes in the phosphorylation levels of Ser 473, they were easily detected in flow cytometry irrespectively of the type of antibody employed. Moreover, we were able to define the sensitivity of Ser 473 p-Akt to either wortmannin or Ly294002. HL-60 cells, derived from human myeloid leukemia, grow as suspension culture, whereas PC-12 cells, derived from a rat pheocromocytoma, grow as adherent culture. However, flow cytometric analysis of Akt was applicable to both cell lines. An interesting finding is that flow cytometric analysis of Ser 473 p-Akt allowed us to establish that in HL-60PT cells the majority of the cells are negative, while only a subset is positive. A similar conclusion could not have been reached by Western blot analysis which just showed that total amount of p-Akt in HL-60PT cells was lower that in AR cells. This is a further proof of the important information which may be gained through the use of flow cytometry.

It has been proposed that the use of antibodies to p-Akt forms in standard pathological techniques such as immunohistochemistry and/or Western blotting might allow valid surrogate measurements of Akt activity in vivo for the screening of lung tumors resistant to chemotherapy and radiotherapy [Brognard et al., 2001].

Taken together, our results show that a quick analysis of total Akt and p-Akt is possible in human cell lines also by means of flow cytometry. We feel that flow cytometric analysis may prove to be useful also for clinical investigation of human neoplasias. In particular, human leukemias and lymphomas are usually subjected to an extensive analysis of morphology

and phenotype to define their degree of differentiation and, consequently, their prognosis [Catovsky and Foa', 1990; Falini and Mason, 2002]. Markers like bcl-2, multiple drug resistance (MDR)-related proteins, and various oncogenes have furthermore been employed to better characterize these neoplastic diseases for defining their susceptibility to therapeutic treatment [Sanz et al., 1997; Kusenda, 1998; Lo Coco et al., 1999; Bannerji and Byrd, 2000; Di Bacco et al., 2000; Misra et al., 2000,]. Since it is increasingly recognized the importance of the PI3K/Akt pathway in determining resistance to chemotherapy [Kaufmann and Gores, 2000; Makin and Hickman, 2000; O'Gorman and Cotter, 2001; Talapatra and Thompson, 2001], the detection of phosphorylated Akt forms could become an additional marker useful to the prognosis and also to devise the best therapeutic approach in several types of human cancers.

REFERENCES

Andjelković M, Suidan HS, Meier R, Frech M, Alessi DR, Hemmings BA. 1998. Nerve growth factor promotes activation of the α, β, and γ isoforms of protein kinase B in PC12 pheocromocytoma cells. Eur J Biochem 251:195–200.

Asselin E, Mills GB, Tsang BK. 2001. XIAP regulates Akt activity and caspase-3-dependent cleavage during cisplatin-induced apoptosis in human ovarian epithelial cancer cells. Cancer Res 61:1862–1868.

Bannerji R, Byrd JC. 2000. Update on the biology of chronic lymphocytic leukemia. Curr Opin Oncol 12:22–29.

Borgatti P, Martelli AM, Bellacosa A, Casto R, Massari L, Capitani S, Neri LM. 2000. Translocation of Akt/PKB to the nucleus of osteoblast-like MC3T3-E1 cells exposed to proliferative growth factors. FEBS Lett 477:27–32.

Bradbury DA, Zhu YM, Russell NH. 1997. Bcl-2 expression in acute myeloblstic leukaemia: Relationship with autonomous growth and CD34 expression. Leukemia Lymphoma 24:221–228.

Brazil DP, Hemmings BA. 2001. Ten years of protein kinase B signalling: A hard Akt to follow. Trends Biochem Sci 11:657–664.

Brognard J, Clark AS, Ni Y, Dennis PA. 2001. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 61: 3986–3997.

Cantrell DA. 2001. Phosphoinositide 3-kinase signaling pathways. J Cell Sci 114:1439–1445.

Catovsky D, Foa' R. 1990. The lymphoid leukaemias. London: Butterworths.

Chan TO, Rittenhouse SE, Tsichlis PN. 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: Kinase activation by phosphoinositide-dependent phosphorylation. Annu Rev Biochem 68:965–1014.

Curti A, Ratta M, Corinti S, Girolomoni G, Ricci F, Tazzari PL, Siena M, Grande A, Fogli M, Tura S, Lemoli RM. 2001. Interleukin-11 induces Th2 polarization of human CD4(+) T cells. Blood 97:2758–2763.

Curti A, Tafuri A, Ricciardi MR, Tazzari PL, Petrucci MT, Fogli M, Ratta M, Lapalombella R, Ferri E, Tura S, Baccarani M, Lemoli RM. 2002. Interleukin-11 induces proliferation of human T-cells and its activity is associated with downregulation of p27(kip1). Haematologica 87:373–380.

Datta SR, Brunet A, Greenberg ME. 1999. Cellular survival: A play in three Akts. Gene Dev 13:2905–2927.

Di Bacco A, Keeshan K, McKenna SL, Cotter TG. 2000. Molecular abnormalities in chronic myeloid leukemia: Deregulation of cell growth and apoptosis. Oncologist 5: 405–415.

Falini B, Mason DY. 2002. Proteins encoded by genes involved in chromosomal alterations in lymphoma and leukemia: Clinical value of their detection by immunocytochemistry. Blood 99:409–426.

Huang P, Oliff A. 2001. Signaling pathways in apoptosis as potential targets for cancer therapy. Trends Cell Biol 11: 343–348.

Kaufmann SH, Gores GJ. 2000. Apoptosis in cancer: Cause and cure. Bioessays 22:1007–1017.

Keng PC, Siemann DW. 1998. Measurement of proliferative activities in human tumor models: A comparison of four flow cytometric methods. Radiat Oncol Invest 6: 120–127.

Kusenda J. 1998. Bcl-2 family proteins and leukemia. Neoplasma 45:117–122.

Lawlor MA, Alessi DR. 2001. PKB/Akt: A key mediator of cell proliferation, survival, and insulin responses? J Cell Sci 114:2903–2910.

Lo Coco F, Diverio D, Avvisati G, Mandelli F. 1999. Diagnosis, front line treatment, and molecular monitoring of promyelocytic leukaemia. Haematologica 84:72–74.

Makin G, Hickman JA. 2000. Apoptosis and cancer chemotherapy. Cell Tissue Res 301:143–152.

Martelli AM, Bortul R, Bareggi R, Grill V, Narducci P, Zweyer M. 1999a. Biochemical and morphological changes in the nuclear matrix prepared from apoptotic HL-60 cells. Effect of different stabilizing procedures. J Cell Biochem 74:99–110.

Martelli AM, Bortul R, Bareggi R, Tabellini G, Grill V, Baldini G, Narducci P. 1999b. The pro-apoptotic drug camptothecin stimulates phospholipase D activity and diacylglycerol production in the nucleus of HL-60 human promyelocytic leukemia cells. Cancer Res 59:3961–3967.

Martelli AM, Bortul R, Fackelmayer F, Tazzari PL, Bareggi R, Narducci P, Zweyer M. 1999c. Biochemical and morphological characterization of the nuclear matrix from apoptotic HL60 cells. J Cell Biochem 72:35–46.

Milosevic D. 2000. Potential diagnostic, prognostic, and therapeutic implication of some new regulatory proteins in hematologic malignancies. J Exp Clin Cancer Res 19: 175–181.

Misra RR, Pinsky PF, Srivastava S. 2000. Prognostic factors for hematologic cancers. Hematol Oncol Clin North Am 14:907–924.

Nicholson KM, Anderson NG. 2002. The protein kinase B/ Akt signalling pathway in human malignancy. Cell Signal 14:381–395.

O'Gorman DM, Cotter TG. 2001. Molecular signals in anti-apoptotic survival pathways. Leukemia 15: 21–34.

Page C, Lin HJ, Jin Y, Castle VP, Nunez G, Huang M, Lin J. 2000. Overexpression of Akt/AKT can modulate chemotherapy-induced apoptosis. Anticancer Res 20: 407–416.

Sanz GF, Sanz MA, Vallespi T. 1997. Etiopathogeny, prognosis, and therapy of myelodisplastic syndromes. Hematol Cell Ther 39:277–294.

Talapatra S, Thompson CB. 2001. Growth factor signalling in cell survival: Implications for cancer treatment. J Pharmacol Exp Ther 298:873–878.

Testa JR, Bellacosa A. 2001. AKT plays a central role in tumorigenesis. Proc Natl Acad Sci USA 98:10983–10985.

Vanhaesebroeck B, Alessi DR. 2000. The PI3K-PDK1 connection: More than just a road to PKB. Biochem J 346:561–576.

Vanhaesebroeck B, Waterfield MD. 1999. Signaling by distinct classes of phosphoinositide 3-kinases. Exp Cell Res 253:239–254.

Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD. 2001. Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem 70:535–602.