The Phosphoinositide 3-Kinase/AKT1 Pathway Involvement in Drug and All-*Trans*-Retinoic Acid Resistance of Leukemia Cells

Luca M. Neri,[1,3] Paola Borgatti,[1] Pier Luigi Tazzari,[4] Roberta Bortul,[5] Alessandra Cappellini,[8] Giovanna Tabellini,[8] Alfonso Bellacosa,[6,7] Silvano Capitani,[1,2] and Alberto M. Martelli[3,8]

[1]Dipartimento di Morfologia ed Embriologia and [2]Interdisciplinary Center for the Study of Inflammation, Università di Ferrara, Ferrara, Italy; [3]Istituto per i Trapianti d'Organo e l'Immunocitologia del C.N.R., Sezione di Bologna, Bologna, Italy; [4]Servizio di Immunoematologia e Trasfusionale, Policlinico S. Orsola, Bologna, Italy; [5]Dipartimento di Morfologia Umana Normale, Università di Trieste, Trieste, Italy; [6]Division of Population Science, Fox Chase Cancer Center, Philadelphia, PA; [7]Dipartimento di Genetica Medica, Università Cattolica del S. Cuore, Rome, Italy; and [8]Dipartimento di Scienze Anatomiche Umane e Fisiopatologia dell'Apparato Locomotore, Università di Bologna, Bologna, Italy

Abstract

Disruption of the apoptotic pathways may account for resistance to chemotherapy and treatment failures in human neoplastic disease. To further evaluate this issue, we isolated a HL-60 cell clone highly resistant to several drugs inducing apoptosis and to the differentiating chemical all-*trans*-retinoic acid (ATRA). The resistant clone displayed an activated phosphoinositide 3-kinase (PI3K)/AKT1 pathway, with levels of phosphatidylinositol (3,4,5) trisphosphate higher than the parental cells and increased levels of both Thr 308 and Ser 473 phosphorylated AKT1. *In vitro* AKT1 activity was elevated in resistant cells, whereas treatment of the resistant cell clone with two inhibitors of PI3K, wortmannin or Ly294002, strongly reduced phosphatidylinositol (3,4,5) trisphosphate levels and AKT1 activity. The inhibitors reversed resistance to drugs. Resistant cells overexpressing either dominant negative PI3K or dominant negative AKT1 became sensitive to drugs and ATRA. Conversely, if parental HL-60 cells were forced to overexpress an activated AKT1, they became resistant to apoptotic inducers and ATRA. There was a tight relationship between the activation of the PI3K/AKT1 axis and the expression of c-IAP1 and c-IAP2 proteins. Activation of the PI3K/AKT1 axis in resistant cells was dependent on enhanced tyrosine phosphorylation of the p85 regulatory subunit of PI3K, conceivably due to an autocrine insulin-like growth factor-I production. Our findings suggest that an up-regulation of the PI3K/AKT1 pathway might be one of the survival mechanisms responsible for the onset of resistance to chemotherapeutic and differentiating therapy in patients with acute leukemia.

Introduction

The development of resistance to a wide spectrum of cytotoxic drugs frequently impedes the successful treatment of acute myeloid leukemias either at the initial presentation or following primary or subsequent relapses (1). Although in the past most of the research on drug resistance focused on the expression of MDR-1/170-kDa P-glycoprotein and the multidrug resistance-associated protein (MRP-1[1]), it is now beginning to emerge that drug resistance is the consequence of failure of leukemic cells to undergo apoptosis in the presence of cytotoxic drugs (2, 3). Indeed, most commonly employed anticancer drugs kill the cells primarily through the induction of apoptosis (1). Remarkable advances in understanding the molecular machinery of apoptosis and the factors initiating the cascade of events leading to apoptotic cell death have been developed in recent years (4). Anti-apoptotic signaling in hematopoietic cells has been associated with a pathway which requires phosphoinositide 3-kinase (PI3K) activity (5). AKT [also known as protein kinase B (PKB)] was identified as a serine/threonine protein kinase downstream of survival signaling through PI3K (6–8). Three different isoforms of Akt have been cloned and named AKT1, AKT2, and AKT3 (7, 8). AKT is activated by a variety of stimuli including growth factors, protein phosphatase inhibitors, and stress (6–9). Several lines of evidence suggest that PI3K regulates AKT1 activation through the binding of phosphatidylinositol (3,4,5) trisphosphate [PtdIns(3,4,5)P_3] to the pleckstrin homology domain of AKT1, resulting in the recruitment of AKT1 to the cell membrane (7). A conformational change of AKT1 then follows allowing residues Thr 308 and Ser 473 to be phosphorylated by upstream kinases. Phosphoinositide-dependent kinase-1 (PDK-1) phosphorylates Thr 308 while Ser 473 is the target of a still unidentified protein kinase (8). Several downstream targets of AKT1 have been identified, pointing to the possible mechanisms by which AKT1 promotes cell survival and blocks apoptosis. There is evidence that AKT1 phosphorylates the pro-apoptotic proteins

Received 6/18/02; revised 12/2/02; accepted 12/3/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Grant support: Italian Miur Cofin 2001 and FIRB 2001 (S.C. and A.M.M.); CARISBO Foundation and Selected Topics Research Fund from Bologna University (A.M.M.); University of Ferrara local funds (L.M.N.); and I.S.S. Progetto Finalizzato Alzheimer and Finanziamento Centro Interuniversitario Biotecnologie (CIB) (S.C.).
Requests for reprints: Alberto M. Martelli, Dipartimento di Scienze Anatomiche Umane e Fisiopatologia dell'Apparato Locomotore, Università di Bologna, via Irnerio 48, 40126 Bologna, Italy. Phone: 39-051-243103; Fax: 39-051-2091695. E-mail: amartell@biocfarm.unibo.it

BAD (on Ser 136), procaspase-9, and transcription factor FKHRL1, resulting in reduced binding of BAD to Bcl-X_L, inhibition of caspase-9 protease activity, and Fas-ligand transcription (7, 8). Moreover, AKT controls the synthesis of anti-apoptotic proteins such as c-IAPs, Bcl-2, and mcl-1 (8). HL-60 cells, derived from a human acute promyelocytic leukemia, are very sensitive to a variety of apoptotic inducers, some of which are employed for the chemotherapeutic treatment of human acute leukemias (2). Moreover, HL-60 cells can be differentiated, *in vitro*, into granulocytes by treatment with all-*trans*-retinoic acid (ATRA) (10). HL-60 cells are known to become resistant to various chemotherapeutic drugs (11) and the occurrence of resistance has, in the past, been ascribed to either MDR-1/170-kDa P-glycoprotein or MRP-1, two members of the ATP-binding cassette superfamily of transporter proteins (12, 13). Recent findings have highlighted that AKT1 is constitutively activated and promotes cellular survival and resistance to chemotherapy in solid tumors (14, 15). However, very limited information is available regarding a possible role played by the PI3K/AKT1 axis in determining multidrug resistance in cells of hematopoietic lineage such as HL-60 cells, even though they frequently acquire a drug-resistant phenotype. Here, we present our data about the characterization of a HL-60 cell clone with an activated PI3K/AKT1 pathway which is resistant to both apoptotic and differentiating stimuli. These cells have high levels of both Thr 308 and Ser 473 phosphorylated AKT1 (p-AKT1). Down-regulation of the PI3K/AKT1 axis with pharmacological inhibitors or overexpression of dominant negative PI3K or AKT1 restored the sensitivity of resistant cells to apoptotic stimuli. Cells displaying enhanced AKT1 activity expressed levels of c-IAP1 and c-IAP2 proteins higher than parental cells. Our results assume a particular significance because they might explain how some acute human leukemias become resistant to cytotoxic drugs as a consequence of chemotherapy.

Results

Isolation and Characterization of an Apoptosis-Resistant Clone of HL-60 Cells

Parental HL-60 (HL-60 PT) cells from the American Type Culture Collection are normally sensitive to a wide variety of apoptotic inducers. A drug-resistant clone was isolated on soft agar and characterized. We named this clone apoptosis resistant HL-60 cells (HL-60 AR cells). We carried out a flow cytometric analysis of some surface markers to characterize these cells. CD13, CD15, CD33, CD63, and CD71 were positive on the large majority of HL-60 PT and AR cells (see Table 1). Analysis of the mean fluorescence intensity showed that there were no differences in the expression of the recognized antigens (data not shown). In contrast, CD3, CD2, CD19, and CD14 were negative on both cell types, thus excluding the presence of contaminating subsets of leukocytes (Table 1). Overall, our results were in agreement with the findings reported by others who thoroughly analyzed the surface phenotype of HL-60 cells (16).

As shown in Table 2, although HL-60 PT cells were highly sensitive to all of the drugs tested, HL-60 AR cells were refractory to all of them. Importantly, drug resistance was seen also after a 24-h incubation, thus leading us to infer that in AR cells, there was not a mere delay of drug-induced apoptosis.

When we measured caspase-3-like activity, an expected increase was measured in HL-60 PT cells in response to either camptothecin or etoposide, whereas there was no activation of the protease activity in HL-60 AR cells (Fig. 1.) We next investigated whether or not HL-60 AR cells were sensitive to ATRA treatment. HL-60 PT cells differentiated up to a percentage of about 80%, using as a parameter of differentiation CD11b expression (Table 3). In contrast, HL-60 AR cells did not respond to the treatment.

PI3K Activity

The PI3K/AKT1 pathway is important in survival, and in some cell types, when activated, it has been shown to block differentiation (17). As presented in Fig. 2A, the amount of three class IA catalytic subunits of PI3K, *i.e.*, p110α, p110β, and p110δ, was similar in the two cell types, as revealed by Western blotting performed on cell homogenates. PI3K was immunoprecipitated from PT or AR HL-60 cells by using an antibody to p85 regulatory subunit. Western blot analysis of the immunoprecipitates showed a similar amount of p85 in HL-60 PT and AR cells. However, if the blots were probed with an antibody to phosphotyrosine after immunoprecipitation with anti-p85 antibody, a marked increase in the immunoreactivity was observed in HL-60 AR cells (Fig. 2B). We next assayed PI3K activity by measuring the levels of PtdIns(3,4,5)P_3 synthesized *in vitro*. In HL-60 AR cells, we measured an increase of approximately 60% in comparison with WT cells. The activity was sensitive to two inhibitors of PI3K, wortmannin (0.1 μM) and Ly294002 (5 μM) (Fig. 2C). We also measured the levels of PtdIns(3,4,5)P_3 synthesized *in vivo* by means of [^{32}P]P_i labeling of intact cells. HL-60 AR cells displayed higher levels of incorporation than HL-60 PT cells. Preincubation of HL-60 AR cells for 24 h with either wortmannin or Ly294002 resulted in lower levels of PtdIns(3,4,5)P_3 (Fig. 2D). To test whether activation of PI3K was relevant to the resistance to various drugs, we used wortmannin and Ly294002. As shown in Table 4, a 24-h incubation in the presence of the inhibitors alone did not increase the percentage of apoptosis. In PT cells, treatment with

Table 1. Surface Marker Analysis of HL-60 PT and AR Cells

Marker	HL-60 PT	HL-60 AR
CD13	93.1 ± 5.4	91.7 ± 7.0
CD15	90.6 ± 6.6	92.2 ± 4.9
CD33	96.6 ± 2.4	95.0 ± 4.0
CD63	89.4 ± 3.2	91.2 ± 5.1
CD71	93.6 ± 4.8	94.9 ± 3.3
CD2	0.5 ± 0.6	0.4 ± 0.7
CD3	0.8 ± 0.4	0.6 ± 0.8
CD14	1.3 ± 1.1	1.5 ± 1.6
CD19	0.6 ± 0.9	0.8 ± 0.9

Note: Cells were stained with FITC-conjugated monoclonal antibodies to surface markers and analyzed by flow cytometry. Data are the means ± SD of three separate experiments and show the percentage of positive cells.

Table 2. HL-60 AR Cells Are Resistant to a Wide Variety of Drugs

Cell Treatment	Percentage of Apoptotic Cells		
	HL-60 PT (6 h)	HL-60 AR (6 h)	HL-60 AR (24 h)
None (control)	4.6 ± 1.4	1.5 ± 1.1	2.1 ± 1.4
Campthotecin (0.1 μg/ml)	64.5 ± 6.2	3.6 ± 1.2	4.5 ± 1.7
Camptothecin (4.0 μg/ml)	89.1 ± 7.0	3.4 ± 2.0	5.4 ± 2.3
Etoposide (5.0 μg/ml)	48.7 ± 5.1	3.5 ± 0.9	4.2 ± 2.1
Etoposide (20 μg/ml)	72.7 ± 5.5	2.4 ± 1.3	3.3 ± 2.6
Cytarabine (40 μg/ml)	42.5 ± 5.4	2.6 ± 1.4	4.9 ± 3.0
Doxorubicin (100 μg/ml)	26.4 ± 2.9	3.1 ± 1.6	5.1 ± 1.8
Mitoxantrone (40 μg/ml)	28.9 ± 3.1	2.8 ± 1.6	4.4 ± 2.3
Vincristine (4 μg/ml)	34.4 ± 3.8	2.9 ± 1.9	4.7 ± 2.9

Note: Cells were exposed for either 6 or 24 h to the drugs. Quantitative analysis of apoptosis was then carried out by flow cytometry. Data are the means ± SD of three separate experiments and show the percentage of apoptotic cells.

either wortmannin or Ly294002 did not result in a significantly higher number of apoptotic cells in response to 6 h drug treatment. In contrast, either of the two inhibitors dramatically increased the number of HL-60 AR cells that underwent apoptosis in response to drugs, even though it did not reach the levels measured in PT cells (compare Table 4 with Table 2).

AKT1 Activity

Western blot analysis with an antibody that recognizes total AKT1 revealed a similar amount of this protein in HL-60 PT and AR cells (Fig. 3A). We next analyzed the amount of the phosphorylated (activated) forms of AKT1 by using antibodies that recognize either Thr 308 p-AKT1 or Ser 473 p-AKT1. As presented in Fig. 3B, the amount of Thr 308 or Ser 473 p-AKT1 was markedly higher in HL-60 AR cells. Such an increase was not evident if the cells had been treated with wortmannin or Ly294002, but not with PD98059 (50 μM), an inhibitor of the mitogen-activated protein kinase pathway. Treatment with either wortmannin or Ly294002 did not lead to a decrease in the level of total AKT1 in HL-60 cells (data not shown).

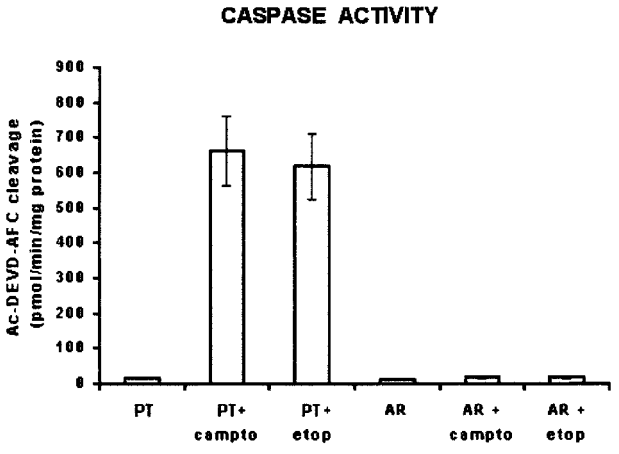

FIGURE 1. Caspase 3-like activity in HL-60 PT and AR cells following treatment with either camptothecin (*campto*) or etoposide (*etop*). *Columns,* means of three experiments; *bars,* SD.

We next assayed AKT1 kinase activity in HL-60 PT or AR cells. As shown in Fig. 3C, we measured a nearly 5-fold increase in AKT1 activity in HL-60 AR cells. Both wortmannin and Ly294002 were capable of inhibiting such an increase.

Transfection Experiments

To further prove that activation of the PI3K/AKT1 axis is important for determining resistance to apoptotic and differentiating stimuli, we performed a series of transfection experiments in HL-60 cells, either PT or AR. We obtained three stably transfected clones of HL-60 AR cells, overexpressing a C-Myc-tagged p110α truncated at its amino terminus (Δp110α). This stretch of 123 amino acids is required for both catalytic activity and interaction with the p85 regulatory subunit of PI3K (18). Western blotting analysis (Fig. 4) showed that the three clones we selected expressed similar amounts of Δp110α. Because we used an antibody to the C-Myc tag, AR cells were negative; Δp110α, when overexpressed, behaved as a dominant negative. Indeed, the three clones exhibited similar levels of *in vitro* PI3K activity which were markedly lower when compared with the PI3K activity of HL-60 AR cells. The activity of mock-transfected cells was comparable to HL-60 AR cell activity (Fig. 4). The three clones had lower levels of Thr 308 p-AKT1 if compared with AR cells (Fig. 4). Also, AKT1 kinase activity in Δp110α overexpressing clones was dramatically lower than in either HL-60 AR cells or mock-transfected cells (Fig. 4). Flow cytometric analysis showed that HL-60 AR cells overexpressing Δp110α (but not mock-transfected cells) regained drug and ATRA sensitivity (Table 5).

We next obtained three stably transfected clones from HL-60 PT cells overexpressing an activated, myristolated form of AKT1, and three stably transfected clones from HL-60 AR cells expressing a dominant negative AKT1. The constitutively active AKT1 is a Gag fusion protein, with an src myristolation signal sequence at its amino terminus. In case of dominant negative AKT1, a mutation (K179M) results in loss of affinity for ATP, and consequently a protein inactive as a kinase. Both the cDNAs are commercially available (see "Materials"). All these clones produced equal levels of the protein of interest, as evidenced by Western blotting analysis (Fig. 5). As presented in Fig. 5, the clones overexpressing constitutively active AKT1 exhibited similar levels of kinase activity, which was higher than

Table 3. Effect of ATRA on HL-60 Cell Differentiation as Evaluated by Means of CD11b Expression and Flow Cytometric Analysis

Cell Treatment	Percentage of Differentiated Cells	
	HL-60 PT	HL-60 AR
None (control)	6.6 ± 1.8	5.7 ± 1.1
24 h ATRA	14.4 ± 2.1	5.8 ± 1.2
48 h ATRA	28.7 ± 3.2	5.9 ± 1.4
72 h ATRA	47.7 ± 4.9	6.1 ± 1.3
96 h ATRA	83.5 ± 6.6	6.4 ± 1.1

Note: Data are expressed as means ± SD of three separate experiments and show the percentage of differentiated cells.

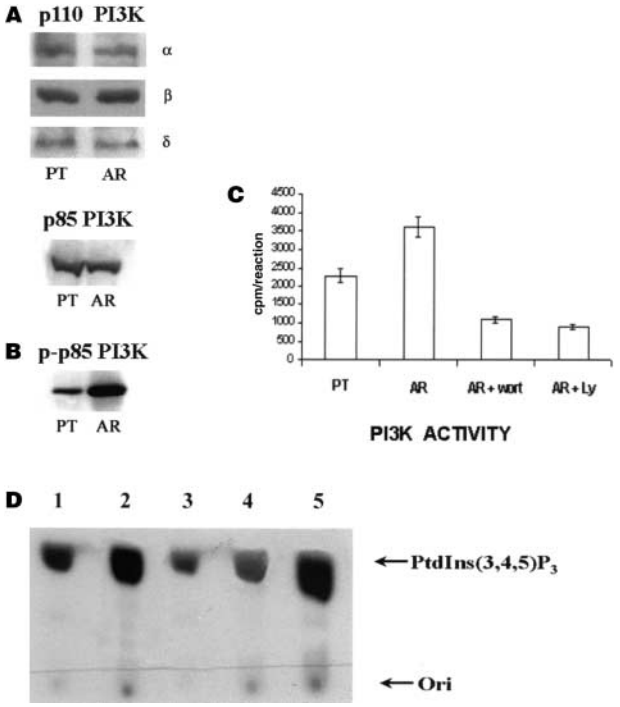

FIGURE 2. PI3K activity is increased in HL-60 AR cells. Western blotting analysis for total p85 and p110 subunits (**A**) and tyrosine-phosphorylated (**B**) p85 subunit of PI3K; 80 μg of protein was blotted to each lane. **C.** *In vitro* PI3K activity in HL-60 PT and AR cells, and in AR cells treated with either 0.1 μM wortmannin (*wort*) or 5 μM Ly294002 (*Ly*) for 24 h. *Columns,* means of three experiments; *bars,* SD. **D.** Representative autoradiograph of a TLC showing *in vivo* PtdIns(3,4,5)P_3 levels in HL-60 PT cells (*lane 1*), HL-60 AR cells (*lane 2*), HL-60 AR cells exposed to 0.1 μM wortmannin (*lane 3*), and HL-60 AR cells exposed to 5 μM Ly294002 (*lane 4*). In this case, cells had been pretreated for 24 h with the inhibitors. PtdIns(3,4,5)P_3 synthesized *in vitro* by human recombinant PI3Kγ, using Phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P_2] as substrate (*lane 5*). *Ori,* origin of the migration. **A**, **B**, and **D** are representative of three separate experiments.

in PT cells. Clones with a forced overexpression of dominant negative AKT1 had levels of kinase activity lower than in AR cells. Clones from PT cells overexpressing constitutively active AKT1 were drug and ATRA resistant, whereas clones from AR cells overexpressing dominant negative AKT1 were sensitive to drugs and ATRA (Table 6). Mock-transfected cells retained the sensitivity of the cells of origin (not shown).

BAD Phosphorylation

The amount of the pro-apoptotic protein BAD was slightly increased in HL-60 AR when compared with HL-60 PT cells (Fig. 6). However, when we used an antibody that specifically recognizes BAD phosphorylated at Ser 136, we found that HL-60 AR cells showed a dramatic increase in this form of phosphorylated BAD (p-BAD). BAD phosphorylation was greatly decreased by treatment with either wortmannin or Ly294002 but not by PD98059.

p70S6K Activity

Another downstream target of PI3K is represented by p70S6K. When samples were probed with an antibody to total

Table 4. Pretreatment with Either Wortmannin or Ly294002 Does Not Enhance the Sensitivity of HL-60 PT Cells While It Restores the Sensitivity of HL-60 AR Cells to the Drugs

Cell Treatment	HL-60 PT (6 h)	HL-60 AR (6 h)
None (control)	4.8 ± 1.5	1.8 ± 0.6
Wortmannin alone (0.1 μM)	5.2 ± 1.7	1.9 ± 1.6
Wortmannin + camptothecin (0.1 μg/ml)	67.3 ± 7.1	50.3 ± 5.9
Wortmannin + etoposide (5 μg/ml)	51.2 ± 6.0	27.6 ± 3.0
Wortmannin + doxorubicin (100 μg/ml)	30.0 ± 3.3	23.4 ± 3.5
Wortmannin + cytarabine (40 μg/ml)	45.1 ± 5.3	32.0 ± 4.1
Wortmannin + mitoxantrone (40 μg/ml)	32.2 ± 3.3	25.5 ± 2.4
Wortmannin + vincristine (4 μg/ml)	36.6 ± 4.2	26.6 ± 2.8
Ly294002 alone (5 μM)	5.2 ± 1.6	2.1 ± 1.4
Ly294002 + camptothecin (0.1 μg/ml)	69.2 ± 7.3	61.4 ± 6.7
Ly294002 + etoposide (5 μg/ml)	50.6 ± 5.9	31.1 ± 3.3
Ly294002 + doxorubicin (100 μg/ml)	31.1 ± 3.4	19.3 ± 2.7
Ly294002 + cytarabine (40 μg/ml)	44.6 ± 4.9	37.7 ± 4.2
Ly294002 + mitoxantrone (40 μg/ml)	31.7 ± 4.0	23.5 ± 2.3
Ly294002 + vincristine (4 μg/ml)	37.7 ± 3.8	28.3 ± 2.9

Note: Incubation with the drugs was for 6 h. Data show the percentage of apoptotic cells and are the means ± SD from three separate experiments.

p70S6K, no important differences were seen between HL-60 PT and AR cells (Fig. 7A), whereas HL-60 AR cells contained a higher amount of phosphorylated p70S6K (p-p70S6K) (Fig. 7B). When we measured *in vitro* p70S6K activity, it became evident

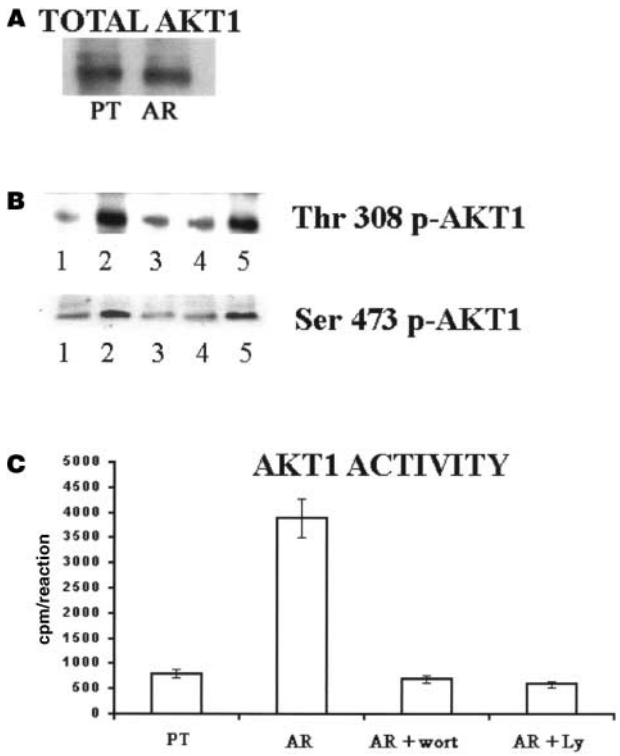

FIGURE 3. AKT1 activity is increased in HL-60 AR cells. Western blotting analysis for total AKT1 (**A**) and Thr 308 or Ser 473 p-AKT1 (**B**). In **B**: *lane 1,* HL-60 PT cells; *lane 2,* HL-60 AR cells; *lane 3,* HL-60 AR cells treated with 0.1 μM wortmannin; *lane 4,* HL-60 AR cells treated with 5 μM Ly294002; and *lane 5,* HL-60 AR cells treated with 50 μM PD98059. Eighty micrograms of protein was blotted to each lane. **C.** AKT1 activity in HL-60 PT or AR cells, and in AR cells treated with either 0.1 μM wortmannin (*wort*) or 5 μM Ly294002 (*Ly*). *Columns,* means of three experiments; *bars,* SD. **A** and **B** are representative of three separate experiments. In **C**, results are the mean of three experiments ± SD.

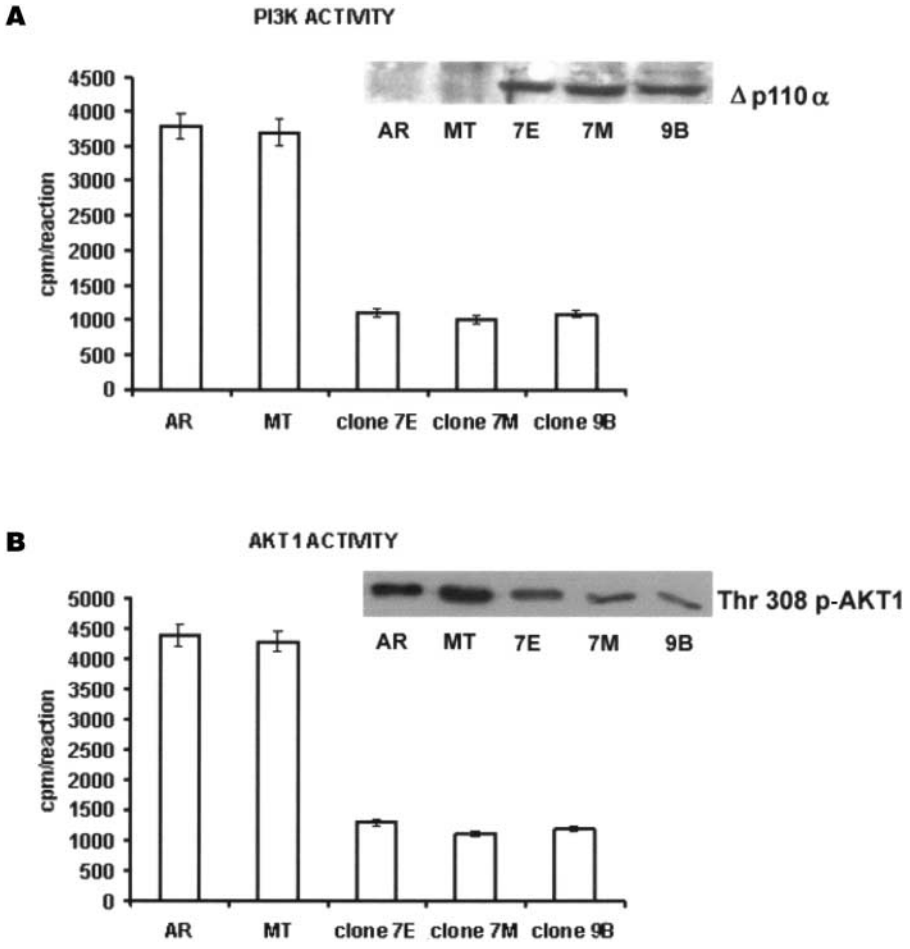

FIGURE 4. *In vitro* PI3K (**A**) and AKT1 (**B**) activities in HL-60 AR cell clones stably overexpressing Δp110α. *Columns,* means of three experiments; *bars,* SD. The *insets* show the results from Western blotting analysis. Eighty micrograms of protein was blotted to each lane. *MT,* mock-transfected clone.

that it was higher in HL-60 AR than in WT cells. The activity was effectively inhibited by pretreating the cells for 4 h with 100 nm of rapamycin, a selective inhibitor of the upstream kinase mammalian target of rapamycin (m/TOR) (Fig. 7C). However, if HL-60 AR cells were exposed to 100 nm rapamycin, we did not detect any increase in the percentage of cells that underwent apoptosis in response to various drugs (Table 7).

Expression of Anti-Apoptotic Proteins

In Fig. 8, we show that the amount of the two anti-apoptotic proteins, c-IAP1 and c-IAP2, was increased in AR when compared with parental cells. Treatment of AR cells with either wortmannin or Ly294002 (but not with PD98059) resulted in lower levels of expression. Higher expression of the two proteins was also found in clone 12FC (constitutively active AKT1), whereas it was lower in clones 7E (dominant negative PI3K) and 2W (dominant negative AKT1). In contrast, the expression of NAIP was similar under all the conditions we tested. Similar results were obtained employing the other stably transfected clones described earlier (data not shown).

PTEN Activity

The increased levels of p-AKT1 in HL-60 AR cells, beyond an increase in PI3K activity, could be due to a decreased phosphatase activity. A major negative regulator of the PI3K/AKT1 pathway is PTEN (19). However, we did not detect any differences in PTEN expression between HL-60 PT and AR cells as revealed by Western blot (Fig. 9A). Furthermore, the *in vitro* PTEN activity was almost the same in both kinds of cells (Fig. 9B).

Increased Tyrosine Phosphorylation of p85 in HL-60 AR Cells Depends on Autocrine Insulin-Like Growth Factor-I Production

We next moved to clarifying the mechanism that leads to enhanced tyrosine phosphorylation of the p85 PI3K regulatory subunit in AR cells. A powerful activator of the PI3K/AKT1 pathway is insulin-like growth factor-I (IGF-I) (*e.g.*, Ref. 20) which exerts an anti-apoptotic function. There is also evidence that HL-60 cells may synthesize and secrete IGF-I, which serves as an autocrine regulator of both myeloid cell growth and differentiation (21). It is well established that IGF-I serves as an endocrine, autocrine, and paracrine stimulator of mitogenesis, survival and carcinogenesis, especially in solid cancers (reviewed in Ref. 22). However, recent evidence indicates that IGF-I can act as an autocrine growth and survival factor in a human myeloma

Table 5. Overexpression of a Dominant Negative p110α (Δp110α) Restores Sensitivity of HL-60 AR Cells to Drugs and ATRA

Treatment	MT[a]	7E	7M	9B
None (control)	1.7 ± 1.3	5.2 ± 2.6	6.1 ± 2.2	5.0 ± 2.3
Camptothecin (0.1 μg/ml)	3.5 ± 2.1	55.0 ± 6.3	64.2 ± 6.8	60.1 ± 6.4
Etoposide (5 μg/ml)	3.8 ± 1.9	39.5 ± 4.6	40.9 ± 4.5	43.7 ± 5.7
Doxorubicin (100 μg/ml)	5.1 ± 2.8	25.0 ± 2.9	28.7 ± 2.8	26.4 ± 3.2
Cytarabine (40 μg/ml)	5.5 ± 2.5	36.3 ± 3.8	41.4 ± 4.6	42.3 ± 5.0
Mitoxantrone (40 μg/ml)	5.6 ± 2.8	26.6 ± 3.0	25.6 ± 3.9	27.4 ± 3.2
Vincristine (4 μg/ml)	5.9 ± 2.7	35.1 ± 3.2	34.5 ± 4.0	31.3 ± 3.6
ATRA	8.1 ± 5.2	76.3 ± 8.1	70.6 ± 8.4	77.7 ± 7.9

Note: The results show the percentage of apoptotic (in the case of drugs) or differentiated (in the case of ATRA) cells. Cells were exposed to the drugs for 24 h or to ATRA for 4 days. Data are the means ± SD from three separate experiments.
[a]MT, mock-transfected clone.

cell line in which IGF-I activated the PI3K pathway (23). Moreover, it should be recalled that childhood acute lymphoblastic leukemia cells have been found to express several components of the IGF-I signaling system, including IGF-I itself (24).

Therefore, we sought to demonstrate that increased tyrosine phosphorylation of p85 seen in HL-60 AR cells was due to a possible autocrine production of IGF-I. As shown in Fig. 10A, reverse transcription (RT)-PCR analysis showed the presence of mRNA coding for IGF-I in exponentially growing AR but not PT cells. We then set out a serum-free system to demonstrate that activation of the PI3K pathway in AR cells is mediated by autocrine secretion of IGF-I. As presented in Fig. 10B, 75% of PT cells were apoptotic after a 48-h serum starvation. In contrast, only 15% of AR cells were apoptotic under the same conditions. The percentage of apoptotic cells did not markedly increase if PT cells were serum starved in the presence of human insulin-like growth factor binding protein-3 (IGFBP-3) (a potent IGF-I antagonist, see Refs. 25 and 26), or an antibody that blocks the binding of IGF-I to its type I receptor, or an antibody which neutralizes the biological activity of IGF-I. On the other hand, IGFBP-3 and either of the two antibodies (but not an irrelevant isotype-matched mouse IgG1) markedly increased the number of apoptotic AR cells under serum-starvation conditions (Fig. 10B). Immunoprecipitation experiments coupled to Western blot revealed that the amount of p85 regulatory subunit of PI3K was the same in serum-starved PT when compared with serum-starved AR cells, even in the presence of IGFBP-3 or of the blocking antibodies. However, tyrosine phosphorylation was much higher in AR than in PT cells and was decreased by IGFBP-3 or the neutralizing antibodies, but not by the irrelevant IgG1 (Fig. 10C). Under serum-starvation conditions, *in vitro* PI3K and AKT1 activities were low in PT cells, whereas in AR cells, about 75% of the activities was retained. In AR cells, PI3K and AKT1 activities were dramatically decreased by incubation of cells in the presence of IGFBP-3, or antibody to type I insulin-like growth factor-I receptor (IGF-IR), or antibody to IGF-I, but not by the irrelevant IgG1 (Fig. 10D).

As shown in Table 8, serum-starved HL-60 AR cells were still resistant to drugs. However, the presence of IGFBP-3 or the blocking antibodies (but not the irrelevant IgG1) rendered cells much more sensitive to drugs.

Discussion

The experiments presented in this article were designed to identify the mechanisms underlying resistance to chemotherapeutic drugs and ATRA in a HL-60 cell clone. Our unpublished experiments had revealed that the amounts of both MDR-1 and MRP-1 were similar in HL-60 AR when compared to PT cells. Moreover, camptothecin is not a substrate of either transporter. Therefore, the involvement of either MDR-1 or MRP-1 in drug resistance seemed unlikely.

We show that PI3K and its downstream target AKT1 are crucial mediators of drug resistance in AR cells, likely via phosphorylation of BAD and/or increased expression of c-IAP1 and c-IAP2. Such a conclusion is based on several findings. In AR cells, we detected increased *in vitro* PI3K activity and enhanced *in vivo* phosphorylation of PtdIns(3,4,5)P_3. This was accompanied by AKT1 phosphorylation at both Thr 308 and Ser 473 and enhanced *in vitro* AKT1 kinase activity. There was a strong correlation between activation of the PI3K/AKT1 axis

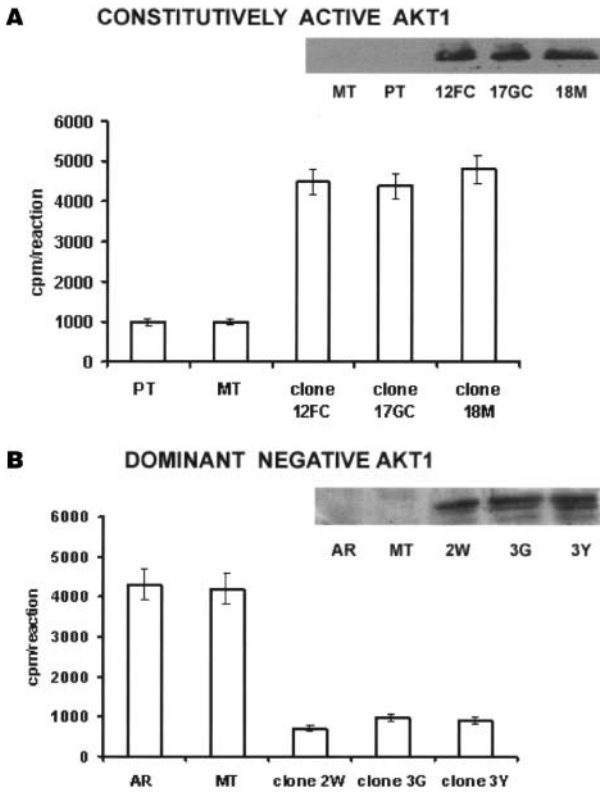

FIGURE 5. *In vitro* AKT1 kinase activity in HL-60 PT cell clones stably transfected with constitutively active AKT1 (**A**) and in HL-60 AR cell clones stably transfected with dominant negative AKT1 (**B**). *Columns,* means of three experiments; *bars,* SD. The *insets* show the results from Western blotting analysis. Eighty micrograms of protein was blotted to each lane. *MT,* mock-transfected clone.

Table 6. Overexpression of a Constitutively Activated AKT1 Renders HL-60 PT Cells Resistant to Apoptosis Inducers and ATRA, Whereas Overexpression of a Dominant Negative AKT1 Restores Sensitivity of HL-60 AR Cells to Drugs and ATRA

Treatment	PT Cells	Transfected PT Cells			AR Cells	Transfected AR Cells		
		12FC	17GC	18M		2W	3G	3Y
None (control)	5.3 ± 1.5	3.7 ± 2.3	3.1 ± 1.8	2.9 ± 2.3	1.5 ± 2.0	5.5 ± 2.1	4.9 ± 2.4	5.7 ± 2.6
Camptothecin (0.1 μg/ml)	77.3 ± 8.9	4.5 ± 2.5	5.0 ± 2.8	4.7 ± 1.8	4.8 ± 2.1	59.5 ± 6.8	63.4 ± 7.0	62.3 ± 7.4
Etoposide (5 μg/ml)	68.2 ± 7.8	4.8 ± 1.9	4.3 ± 1.6	4.9 ± 1.9	4.6 ± 1.8	37.7 ± 4.1	41.2 ± 4.4	43.5 ± 4.3
Doxorubicin (100 μg/ml)	46.6 ± 6.1	5.0 ± 3.8	5.9 ± 2.2	4.7 ± 2.1	5.6 ± 2.3	25.4 ± 3.1	24.9 ± 3.6	23.6 ± 3.3
Cytarabine (40 μg/ml)	59.4 ± 5.6	6.5 ± 2.4	6.7 ± 3.0	5.4 ± 2.6	5.2 ± 1.7	40.8 ± 4.6	42.1 ± 5.0	44.5 ± 5.1
Mitoxantrone (40 μg/ml)	51.5 ± 6.6	5.5 ± 2.5	6.0 ± 2.5	5.6 ± 2.9	4.7 ± 2.6	28.3 ± 3.6	29.5 ± 4.0	26.6 ± 3.7
Vincristine (4 μg/ml)	54.4 ± 6.0	4.9 ± 2.6	5.7 ± 2.2	4.9 ± 2.7	4.4 ± 3.0	32.2 ± 3.7	32.8 ± 3.4	30.3 ± 2.9
ATRA	79.6 ± 8.0	7.3 ± 3.1	6.9 ± 3.1	6.6 ± 3.4	7.1 ± 1.4	75.9 ± 7.3	72.3 ± 8.1	78.7 ± 8.8

Note: The results show the percentage of apoptotic (in the case of drugs) or differentiated (in the case of ATRA) cells. Cells were exposed to the drugs for 24 h or to ATRA for 4 days. Data are the means ± SD from three separate experiments.

and the drug-resistant phenotype of HL-60AR cells: prolonged incubation with wortmannin or Ly294002 of AR cells led to decreased PI3K and AKT1 activity and resulted in restoration of the drug-sensitive phenotype. Furthermore, overexpression of a constitutively active AKT1 rendered PT cells resistant to chemotherapeutic drugs and ATRA, whereas AR cells with a forced overexpression of either dominant negative p110α or dominant negative AKT1 regained sensitivity to drugs and ATRA.

HL-60AR cells had a slightly elevated amount of total BAD when compared with WT cells. However, there was a dramatic increase in the levels of Ser 136 p-BAD, which was very sensitive to treatment with PI3K inhibitors. As far as c-IAP1 and c-IAP2 proteins are concerned, their levels of expression were in tight relationship with the AKT1 activity levels. These proteins antagonize caspase-3 activation and activity (4), and their expression is regulated by the PI3K/AKT1 survival pathway through the action of NFκ-B (8).

Another downstream target of PI3K that we have investigated is p706SK. The activity of this kinase is important for cell survival signaling and it has recently been reported that p70S6K is capable of phosphorylating BAD at Ser 136 (27). This may explain its anti-apoptotic effect. Phosphorylation of p70S6K correlates with its activity (*e.g.*, Ref. 28). Although in HL-60 AR cells we observed an increased *in vitro* activity of p70S6K as well as its hyperphosphorylation when compared with PT cells, treatment of AR cells with rapamycin (which effectively reduced *in vitro* p70S6K activity) did not restore sensitivity to drugs. Therefore, an involvement of p70S6K in drug resistance seems unlikely.

The results presented in this article highlight the important role played by the PI3K/AKT1 pathway in preventing chemotherapy-induced apoptosis. As far as we know, this is the first report that clearly links multidrug and ATRA resistance of HL-60 cells with the PI3K/AKT1 survival pathway. Our results are in part consistent with and complement those reported recently by O'Gorman *et al.* (2) who showed that, by inhibiting the PI3K surviving signals, HL-60 cells were sensitized to cytotoxic drug-induced apoptosis. However, we would like to underscore that our findings differ from theirs. Indeed, they did not select a drug-resistant clone but rather performed the experiments on cells presumed to be parental and showed an enhancement of drug sensitivity on treatment with PI3K inhibitors, whereas we also performed transfection experiments to demonstrate specificity for the PI3K/AKT1 pathway. However, we did not observe an enhanced drug sensitivity of our PT cells on treatment with PI3K inhibitors. They also came to the conclusion that BAD phosphorylation on Ser-136 does not depend on the survival pathway controlled by PI3K/AKT1. Moreover, we investigated the effect of an up-regulation of the PI3K/AKT1 axis on an important molecule such as ATRA.

What are the mechanisms leading to increased PI3K activity in AR cells? In principle, it might be argued that aberrant phosphatase function contributes to AKT1 activation in AR cells. The 3′ phosphoinositide phosphatase PTEN has been shown to regulate AKT1 activity (29). However, our data revealed that both the amount of PTEN and its *in vitro* activity were similar in HL-60 WT and AR cells. Thus, we feel it is unlikely that enhanced AKT1 activity seen in AR cells is dependent on a decrease in PTEN function. It has been reported that HL-60 cells either are completely devoid of PTEN (30) or express a nonfunctional PTEN that lacks exons 2–5 (31). We would like to underscore that, by Western blot analysis, we

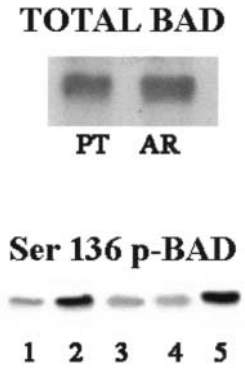

FIGURE 6. Western blotting analysis for total (*upper panel*) and Ser 136 p-BAD (*lower panel*) in HL-60 PT and AR cells. In *lower panel*: *lane 1,* HL-60 PT cells; *lane 2,* HL-60 AR cells; *lane 3,* HL-60 AR cells treated with 0.1 μM wortmannin; *lane 4,* HL-60 AR cells treated with 5 μM Ly294002; and *lane 5,* HL-60 AR cells treated with 50 μM PD98059. HL-60 AR cells had been incubated with the inhibitors for 24 h. Eighty micrograms of protein was blotted to each lane. Results are representative of three separate experiments.

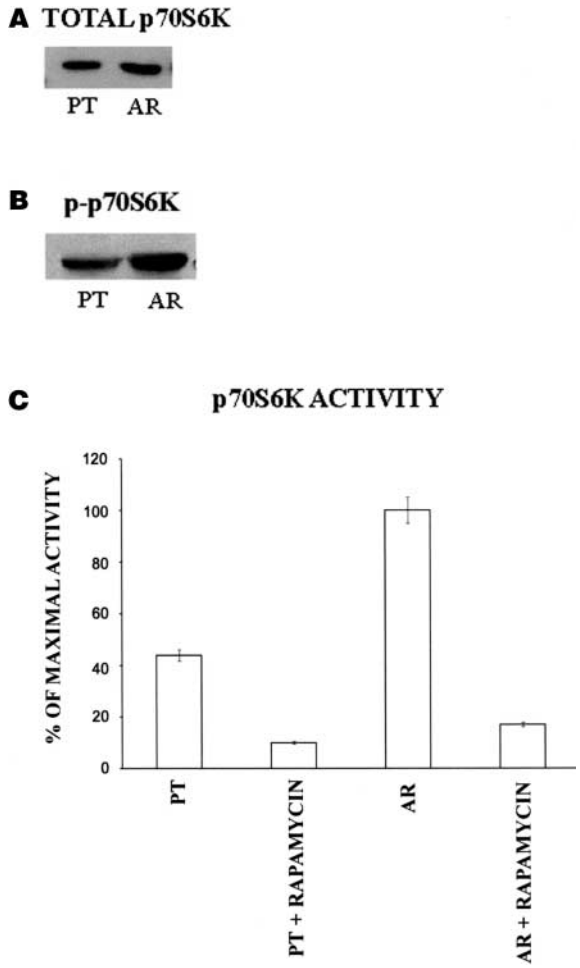

FIGURE 7. p70S6K activity is increased in HL-60 AR cells. Western blotting analysis for total (**A**) and p-p70S6K (**B**). Eighty micrograms of protein was blotted to each lane. **C.** *In vitro* p70S6K activity in HL-60 PT or AR cells in the absence and in the presence of 100 nm rapamycin; cells had been pretreated with rapamycin for 4 h. *Columns,* means of three experiments; *bars,* SD. **A** and **B** are representative of three separate experiments. One hundred percent of the activity was 3327 ± 305 cpm/reaction.

always detected PTEN at its expected molecular weight in our cells and that we were able to assay its *in vitro* activity. Thus, we should not rule out the possibility that previous investigations (30, 31) dealt with particular clones of HL-60 that either completely lacked or had a nonfunctional PTEN.

Our results point to a primary role of increased tyrosine phosphorylation in AR cells. The amount of PI3K p85, p110α, p110β, and p110δ subunits did not vary between HL-60PT and AR cells, as demonstrated by Western blotting analysis. In contrast, we detected a marked increase in the tyrosine phosphorylation status of p85 subunit in AR cells. This was accompanied by an increased *in vitro* PI3K activity and by an enhanced *in vivo* phosphorylation of PtdIns(3,4,5)P_3. These findings are consistent with recent observations (32, 33) indicating that a hyperphosphorylation on tyrosine residues of the p85 regulatory subunit of PI3K accompanies activation of this enzyme. Our data seem to indicate that in HL-60 AR cells, there is an autocrine production of IGF-I and this growth factor is responsible for increased tyrosine phosphorylation of p85 and up-regulation of the PI3K/AKT1 axis. Indeed, we detected IGF-I mRNA in AR but not in PT cells. AR cells were much more resistant than PT cells to a 48-h serum starvation. However, the percentage of apoptotic cells increased when AR cells were serum starved in the presence of IGFBP-3 or of antibodies that blocked either the IGF-IR or IGF-I itself. IGF-BP3 or the blocking antibodies markedly reduced tyrosine phosphorylation of p85 as well as PI3K and AKT1 activities. Moreover, the presence of IGFBP-3 or of the blocking antibodies rendered serum-starved HL-60 AR cells drug sensitive. Our HL-60 AR cells were originally selected from cells that survived a 48-h exposure to either camptothecin or etoposide. It is beginning to emerge that, in some cases, the very stimulus that produces apoptosis also initiates an antagonistic anti-apoptotic program (34). For example, it has been previously reported that daunorubicin rapidly activates the PI3K/AKT pathway in U937 human leukemia cells, but the mechanism responsible for such an activation was unclear (35). We suggest that prior exposure to either camptothecin or etoposide positively selected IGF-I-producing cells. Therefore, our findings may provide a further insight into the molecular mechanisms of "inducible multidrug resistance" (34).

A very important observation is that HL-60AR cells are resistant not only to chemotherapeutic agents but also to granulocytic differentiation induced by ATRA. In acute promyelocytic leukemia, the addition of ATRA to chemotherapy has increased the percentage of long-term survivors from 40% to 70% (36). We are at present investigating the molecular mechanisms, mediated by PI3K/AKT1, underlying ATRA resistance in HL-60 AR cells. In any case, our results are in total agreement with the findings reported very recently by others who showed that AKT mediates ATRA resistance in breast cancer cells (37).

Our results may have important implications for future preclinical and clinical studies. Indeed, they might pave the way for investigations aimed at determining whether or not activation of the PI3K/AKT1 pathway is responsible for drug and ATRA resistance of patients suffering from acute promyelocytic leukemia. The fact that the PI3K/AKT1 pathway promotes resistance to several drugs and to a differentiating

Table 7. Rapamycin Does Not Enhance the Sensitivity of HL-60 AR Cells to Apoptosis Inducers

Cell Treatment	Percentage of Apoptotic Cells
None (control)	1.8 ± 0.9
Rapamycin alone	2.9 ± 1.2
Rapamycin + camptothecin (0.1 μg/ml)	2.7 ± 1.4
Rapamycin + etoposide (5 μg/ml)	3.4 ± 1.6
Rapamycin + doxorubicin (100 μg/ml)	3.6 ± 1.5
Rapamycin + cytarabine (40 μg/ml)	2.8 ± 1.7
Rapamycin + mitoxantrone (40 μg/ml)	3.0 ± 1.5
Rapamycin + vincristine (4 μg/ml)	3.4 ± 1.9

Note: The data are the means ± SD from three separate experiments. Cells were exposed to the drugs for 24 h.

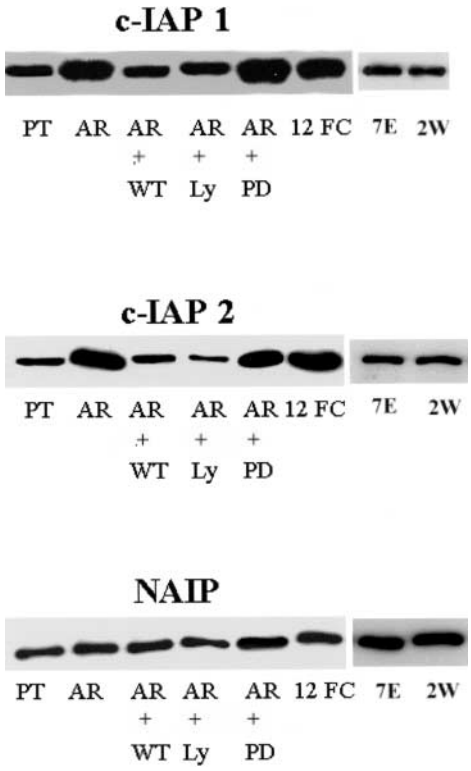

FIGURE 8. Western blotting analysis for c-IAP1, c-IAP2, and NAIP in HL-60 PT, HL-60 AR, and clones 12FC, 7E, and 2W. When the pharmacological inhibitors were used, cells had been preincubated for 24 h with nontoxic concentrations of wortmannin (0.1 μM, *WT*), Ly294002 (5 μM, *Ly*), and PD98059 (50 μM, *PD*). Eighty micrograms of protein was blotted to each lane. Results are representative of three separate experiments.

agent such as ATRA in acute human leukemia-derived cells might also gain predictive value for response to chemotherapy and/or ATRA treatment, so that in the future, evaluation of the PI3K/AKT1 pathway might even become of prognostic relevance in this disease, even though the involvement of other survival pathways, such as the mitogen-activated protein kinase pathway will have to be considered. Utilization of antibodies to phosphorylated forms of AKT1 in standard pathological techniques such as immunohistochemistry and/or Western blotting might allow valid surrogate measurements of AKT1 activity *in vivo* and guide clinical management of leukemia. In the future, we can envision the clinical use of pharmacological inhibitors specific for PI3K isoforms or AKT1 to restore sensitivity of leukemic cells to both chemotherapy and differentiating agents.

Methods

Materials

BSA, etoposide, camptothecin, vincristine, cytarabine, G418, protein A/G agarose, human recombinant PI3K (p110γ), human recombinant IGFBP-3, anti-human IGF-I, isotype-matched mouse IgG1, and peroxidase-conjugated secondary antibodies were obtained from Sigma, St. Louis, MO. Histone H2B, COMPLETE Protease Inhibitor Cocktail, and the Lumi-Light^{Plus} enhanced chemiluminescence detection kit were from Roche Applied Science, Milan, Italy. Constitutively active and dominant negative AKT1 cDNAs (both cloned in pUSEamp and C-Myc tagged), p70S6K, and PTEN assay kits were from Upstate Biotechnology, Lake Placid, NY. PtdIns(4,5)P_2, Ly 294002, wortmannin, PD98059, the caspase-3 fluorimetric substrate *N*-acetyl-Asp-Glu-Val-aspartyl-aminofluoro-methyl-coumarine (Ac-DEVD-AFC), mitoxantrone, doxorubicin, ATRA, rapamycin, and C2 ceramide were obtained from Calbiochem, La Jolla, CA. PtdIns(3,4,5)P_3 was from Alexis Biochemical, Laufelfingen, Switzerland. The Protein Assay kit (detergent compatible) and DTT were from Bio-Rad, Hercules, CA. cAMP-dependent protein kinase inhibitor peptide was obtained from Bachem (Bubendorf, Switzerland). P-81 paper was procured from Whatman (Maidstone, UK). QIAshedder, RNeasy, RNase free DNase set, Omniscript reverse transcriptase, and Taq polymerase were acquired from Qiagen, Milan, Italy. Mouse Δp110α cDNA, cloned in pUSEamp and C-Myc tagged, was a kind gift from Dr. S. Marmiroli (Istituto di Citomorfologia Normale e Patologica del CNR, Bologna, Italy).

Source of Antibodies

The following primary antibodies were used in this study. From Upstate Biotechnology: rabbit polyclonals to p85 regulatory subunit of PI3K, to p110β catalytic subunit of PI3K, to total p70S6K, to total AKT1, and mouse monoclonal antibody to phosphotyrosine (clone 4G10). From Santa Cruz Biotechnology (Santa Cruz, CA): rabbit polyclonals to c-IAP1 and c-IAP2, mouse monoclonals to Ser 411 p-p70S6K and c-Myc (clone 9E10), goat polyclonal to NAIP. From Cell

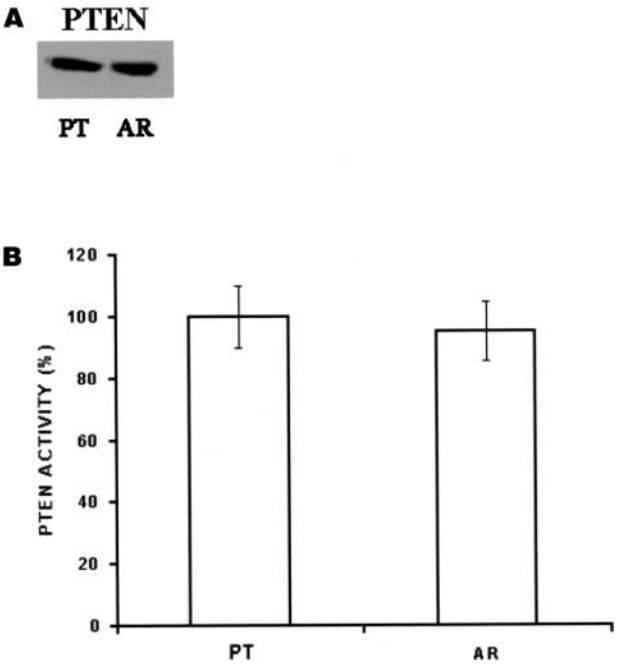

FIGURE 9. Analysis of PTEN. Expression (**A**) and *in vitro* activity (**B**) of PTEN in HL-60 PT and AR cells. Results in **A** are representative of three separate experiments; in **B**: *columns,* means of three experiments; *bars,* SD. In **A**, 80 μg of protein was blotted to each lane.

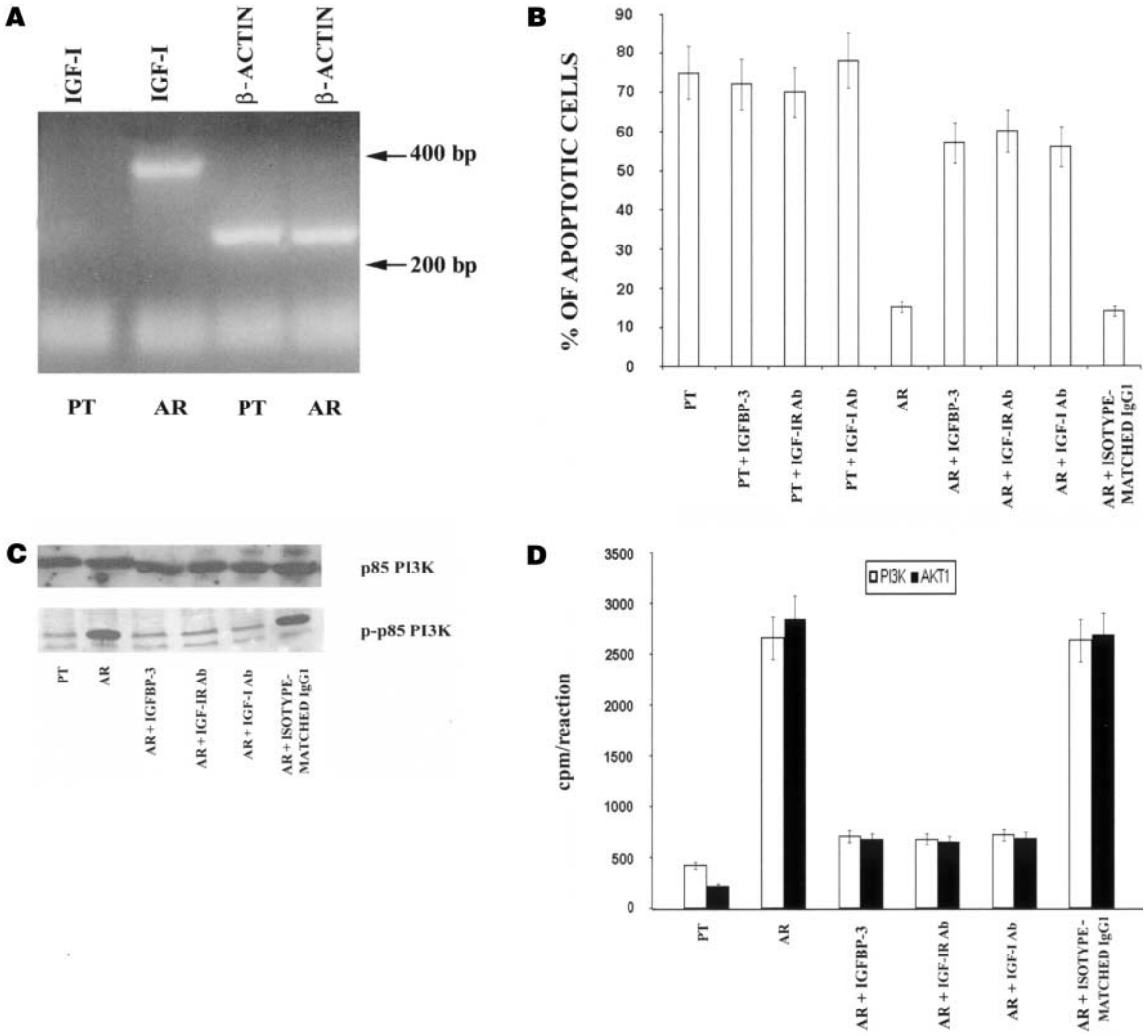

FIGURE 10. An autocrine production of IGF-I is responsible for enhanced tyrosine phosphorylation and PI3K activity in HL-60 AR cells. **A.** RT-PCR analysis showing the expression of the IGF-I gene in exponentially growing HL-60 AR cells. **B.** Flow cytometric analysis showing the percentage of apoptotic cells in HL-60 PT and AR cells incubated for 48 h in serum-free RPMI 1640 containing 0.5% BSA. IGFBP-3 was present at 500 ng/ml, whereas the antibodies were present at 4 μg/ml (see Ref. 20). **C.** Western blot analysis showing the levels of total and tyrosine-phosphorylated p85 subunit of PI3K. For p85 analysis, the regulatory subunit of PI3K was immunoprecipitated from cell homogenates, electrophoresed, blotted, and then revealed by antibodies to either p85 or phosphotyrosine. **D.** PI3K and AKT1 activities in PT cells and AR cells serum starved for 48 h. Concentrations of IGFBP-3 and of the antibodies were as reported in **B**. In **B** and **D**: *columns,* means of three experiments; *bars,* SD.

Signaling Technology (Beverly, MA): mouse monoclonal to PTEN, rabbit polyclonals to Thr 308 p-AKT1, Ser 473 p-AKT1, total BAD, Ser 136 p-BAD. From Calbiochem: rabbit polyclonal to p110δ catalytic subunit of PI3K and mouse monoclonal to IGF-IR. From BD Biosciences Transduction Laboratories, Franklin Lakes, NJ: mouse monoclonal to p110α catalytic subunit of PI3K. From Sigma: monoclonal antibody to β-tubulin.

Cell Culture and Differentiation

The HL-60 cell line, obtained from the American Type Culture Collection (CCL 240), was routinely maintained in RPMI 1640 supplemented with 10% FCS at an optimal cell density of 3 to 8 $\times$ 10^5 cells/ml. Exponentially growing HL-60 cells were seeded in fresh media at a concentration of 5 $\times$ 10^5 cells/ml in the presence or in the absence of ATRA (from a stock solution in DMSO). After 96 h, differentiation was evaluated by flow cytometric analysis of CD11b expression (10).

Isolation of a Variant HL-60 Cell Clone

During our previous studies on apoptosis (38–40), we fortuitously selected a HL-60 cell population that did not die after a 48-h incubation in the presence of two powerful apoptosis inducers, such as camptothecin and etoposide. These drug-resistant HL-60 cells were diluted at a density of about 100 cells for 10 ml of filtered, conditioned RPMI 1640. 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.

Induction of Apoptosis and Flow Cytometric Analysis

Sixteen hours after medium change, HL-60 cells in exponential growing phase were treated with apoptotic inducers for either 6 or 24 h at 37°C. In some experiments, before apoptotic induction, cells had been preincubated for 24 h with nontoxic concentrations of wortmannin (0.1 μM) or Ly294002 (5 μM). For detection of apoptosis, samples containing 2 to 5 × 10^5 cells were harvested by centrifugation at 200 × *g* for 10 min, fixed with 70% cold ethanol for 1 h, and stained with 20 μg/ml propidium iodide for 30 min at room temperature. For quantitative evaluation of apoptosis, the subdiploid DNA content was calculated as described by Nicoletti *et al.* (41) using an Epics XL flow cytometer with the appropriate software (Beckman-Coulter, Hialeah, FL). To evaluate the phenotype of HL-60 cells, we used monoclonal antibodies to the following antigens, according to Trayner *et al.* (16): CD13, CD15, CD33, CD63, and CD71. Moreover, we also stained cells with monoclonal antibodies to CD3, CD2, CD19, and CD14, to exclude a possible contamination by different subsets of leukocytes. All the antibodies were purchased from Beckman-Coulter, as FITC conjugates. Staining was performed as described by Trayner *et al.* (16). Control samples were run with irrelevant, FITC-conjugated mouse IgGs of the same isotype of the antibodies employed. Analysis was performed by an EPICS-XL flow cytometer.

In Vivo Labeling of Polyphosphoinositides, Lipid Extraction, and TLC

Cells were washed once with PBS and incubated with phosphate-free RPMI 1640/0.5% BSA for 15 min, then exposed to the same medium plus carrier-free [^{32}P]P_i (1.0 mCi/ml) at 37°C for 8 h. Cells (2 × 10^7), washed twice with ice-cold buffer A [137 mM NaCl, 20 mM Tris (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM Na_3VO_4], were transferred to a tube containing 3 ml of chloroform/methanol (1:2) plus 1 mg/ml of butylated hydroxytoluene and 5 μg of PtdIns(4,5)P_2. After addition of 2.1 ml of chloroform and 2.1 ml of 2.5 M HCl, the lower phase was collected, and the upper phase was washed twice with 1 ml of chloroform. The three upper phases were pooled and dried under vacuum. The lipids were spotted on a silica plate and separated by using chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14). Plates were autoradiographed and the position of the products was compared with migration of both unlabeled PtdIns(3,4,5)P_3 and labeled PtdIns(3,4,5)P_3 synthesized *in vitro* by human recombinant PI3K (p110γ). Spots of interest were scraped and counted by liquid scintillation.

Cell Transfection

Transfection of HL-60 cells was performed by electroporation with a Bio-Rad Gene Pulser apparatus. Twenty micrograms of plasmid DNA were mixed with 10^7 cells in 0.5 ml of phosphate-buffered sucrose [272 mM sucrose and 7 mM Na_2HPO_4 (pH 7.4)]. Cells were electroporated with a pulse of 250 V for 18–20 ms. Control cells were mock transfected in the same conditions with the empty vector. Following electroporation, cells were allowed to recover in 20 ml of culture medium for 48 h before selection with 600 μg/ml of G418. G418-resistant transfected clones were obtained by limited dilution.

Caspase-3-like activity was measured by cleavage of Ac-DEVD-AFC exactly as described by others (42).

Western Blot Analysis

To prepare all homogenates for Western blot analysis, they were washed twice in PBS containing the COMPLETE Protease Inhibitor Cocktail supplemented with 1.0 mM Na_3VO_4 and 20 nM okadaic acid and were then lysed at ~10^7/ml in boiling electrophoresis sample buffer containing the protease and phosphatase inhibitor cocktail. Lysates were briefly sonicated to shear DNA and reduce viscosity and boiled for 5 min to solubilize protein.

Protein separated on SDS-polyacrylamide gels was transferred to nitrocellulose sheets using a semi-dry blotting apparatus. Bands were visualized by the enhanced chemiluminescence method. To ensure equal loading, blots were always first probed with an antibody to β-tubulin, then stripped and reprobed.

Immunoprecipitation

To prepare cell homogenates for immunoprecipitation, 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.

Table 8. Drug Sensitivity of HL-60 AR Cells After a 48-h Serum Starvation

Treatment	AR Cells	AR Cells + IGFBP-3	AR Cells + IGF-IR Ab	AR Cells + IGF-I Ab	AR Cells + Isotype-Matched IgG1
None (control)	14.7 ± 2.3	5.2 ± 2.6	6.1 ± 2.2	5.0 ± 2.3	15.9 ± 2.1
Camptothecin (0.1 μg/ml)	20.5 ± 3.4	95.0 ± 9.3	94.2 ± 8.8	90.1 ± 9.4	22.5 ± 3.7
Etoposide (5 μg/ml)	19.5 ± 1.9	79.5 ± 8.8	81.9 ± 8.6	83.4 ± 9.7	21.3 ± 2.2
Cytarabine (40 μg/ml)	15.1 ± 3.3	75.0 ± 7.9	78.7 ± 7.7	80.4 ± 8.2	15.7 ± 2.9
Doxorubicin (100 μg/ml)	15.8 ± 2.4	75.5 ± 8.8	81.4 ± 9.5	86.3 ± 9.0	16.9 ± 2.6
Mitoxantrone (40 μg/ml)	15.7 ± 4.0	86.4 ± 8.9	87.6 ± 8.4	80.4 ± 9.9	16.4 ± 3.1
Vincristine (4 μg/ml)	16.7 ± 2.7	80.1 ± 9.2	81.5 ± 8.0	77.8 ± 8.8	17.3 ± 2.3

Note: Cells were incubated with the drugs for 6 h. The results show the percentage of apoptotic cells and are the means ± SD of three separate experiments.

Cell homogenates (1 ml, containing 500 μg of protein) were precleared by adding 5 μg of normal rabbit IgG and 10 μg of 50% protein A/G 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 the antibody to p85 regulatory subunit of PI3K, AKT1, p70S6K, or PTEN. Ten micrograms of 50% protein A/G agarose was then added and incubation proceeded for 1 h at 4°C under constant agitation.

PI3K Activity Assay

The immunoprecipitates were washed twice with each of the following buffers: (*a*) PBS (pH 7.4) containing 1% Nonidet P-40; (*b*) 100 mM Tris-HCl (pH 7.4) and 0.5 M LiCl; and (*c*) 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM EDTA. All washing solutions contained 1 mM Na_3VO_4. The PI3K activity assay was then performed by adding sonicated PtdIns(4,5)P_2 [0.5 mg/ml in 10 mM HEPES-NaOH (pH 7.5) and 1 mM EDTA], 10 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP (10 Ci/mmol). Incubation was for 15 min at room temperature. The reaction was stopped by addition of chloroform/methanol/HCl (200:100:0.75, v/v), followed by two washes with chloroform/methanol/HCl 0.6 N (3:48:47). The lipid-containing organic phase was resolved on TLC plates developed in isopropanol:-acetic acid:H_2O (65:1:34). The radiolabeled PtdIns(3,4,5)P_3 was identified by comparison with standard PtdIns(3,4,5)P_3. After autoradiography, the spots were excised and quantified by scintillation counting.

AKT1 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 (43). 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, and 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol). Samples were incubated for 30 min at 30°C and the reaction was then stopped by spotting 80 μl onto 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 anti-AKT1 antibody was replaced by normal rabbit IgG, were subtracted from all values.

p70S6K and PTEN Activity Assay

For p70S6K activity, immunoprecipitation products were washed twice in lysis buffer followed by a wash in kinase buffer [20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mg ml BSA, 0.4 mM DTT]. The kinase reaction was performed for 15 min at 30°C with provided S6 peptide pseudosubstrate (125 μM) in the presence of 100 μM ATP and 0.06 μM [γ-^{32}P]ATP. The reaction was stopped by spotting samples onto Whatman P-81 filter papers and immersing in 1% (v/v) orthophosphoric acid. Then, the procedure was continued as for PI3K activity assays.

For PTEN activity, the immunoprecipitates were washed in a buffer containing 20 mM HEPES-NaOH (pH 7.7), 50 mM NaCl, 0.1 mM EDTA, and 2.5 mM $MgCl_2$, followed by a wash in phosphatase assay buffer lacking PtdIns(3,4,5)P_3 [100 mM Tris-HCl (pH 8.0) and 10 mM DTT]. Samples were incubated in a 50-μl volume containing mixed phospholipid vesicles [final concentrations: 415 μg/ml dioleyl phosphatidylcholine, 415 μg/ml dioleyl phosphatidylserine, 200 μg/ml PtdIns(3,4,5)P_3 in 10 mM HEPES-NaOH (pH 7.4), 1 mM EGTA, 1 mg/ml BSA]. Reactions proceeded for 15 min at 37°C and were stopped by the addition of 200 μl of malachite green solution. Absorbance was measured at 640 nm. Phosphate standard curves were obtained by employing a recombinant PTEN-GST fusion human protein.

RT-PCR

Total RNA was extracted with the QIAshedder, RNeasy, and RNase free DNase set according to the manufacturer's protocol. Two-microgram samples of total RNA were reversed transcribed into cDNA by Omniscript reverse transcriptase, as recommended by the manufacturer. Aliquots of the cDNA were used for enzymatic amplification in 50 μl reactions with 1 unit Taq polymerase and 0.2 pmol of appropriate gene-specific primers in a Perkin-Elmer Gene Amp PCR System 2400. All RT-PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gel and photographed. The following primers and PCR conditions were used. (The genes are given with database accession number and primer localization.)

(*a*) β-actin (M10277): 5′ primer: nucleotides (nt) 1131–1148, 3′ primer: nt 2888–2869 (95°C for 3 min, then 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min), and
(*b*) IGF-I (X00173): 5′ primer: nt 16–39, 3′ primer: nt 413–391 (cycling conditions as for β-actin).

Acknowledgments

The authors thank Anna Maria Billi for help with the illustrations.

References

1. O'Gorman, D. M. and Cotter, T. G. Molecular signals in anti-apoptotic survival pathways. Leukemia, *15:* 21–34, 2001.

2. O'Gorman, D., McKenna, S. L., McGahon, A. J., Knox, K. A., and Cotter, T. G. Sensitisation of HL60 human leukaemic cells to cytotoxic drug induced apoptosis by inhibition of PI3-kinase survival signal. Leukemia, *14:* 602–611, 2000.

3. Kaufmann, S. H. and Earnshaw, W. C. Induction of apoptosis by cancer chemotherapy. Exp. Cell Res., *256:* 42–49, 2000.

4. Hengartner, G. The biochemistry of apoptosis. Nature, *407:* 770–776, 2000.

5. Minshall, C., Arkin, S., Freund, G. G., and Kelley, K. W. Requirement for phosphatidylinositol 3′-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. J. Immunol., *156:* 939–947, 1996.

6. Datta, S. R., Brunet, A., and Greeberg, M. E. Cellular survival: a play in three Akts. Genes Dev., *13:* 2905–2907, 1999.

7. Brazil, D. P. and Hemmings, B. A. Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem. Sci., *26:* 657–664, 2001.

8. Nicholson, K. M. and Anderson, N. G. The protein kinase B/Akt signaling pathway in human malignancy. Cell Signal., *14:* 381–395, 2002.

9. Lawlor, M. A. and Alessi, D. R. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? J. Cell Sci., *114:* 2903–2910, 2001.

10. Collins, S. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood, *70:* 1233–1244, 1987.

11. Marks, D. C., Su, G. M., Davey, R. A., and Davey, M. W. Extended multidrug resistance in haemopoietic cells. Br. J. Haematol., *95:* 587–595, 1996.

12. Palissot, V., Belhoussine, R., Carpentier, Y., Sebille, S., Morjani, H., Manfait, M., and Dufer, J. Resistance to apoptosis induced by topoisomerase I inhibitors in multidrug-resistant HL60 leukemic cells. Biochem. Biophys. Res. Commun., *245:* 918–922, 1998.

13. Wada, H., Saikawa, S., Niida, Y., Nishimura, R., Noguchi, T., Matsukawa, H., Ichihara, T., and Koizumi, S. Selectively induced high MRP gene expression in multidrug-resistant human HL60 leukemia cells. Exp. Hematol., *27:* 99–109, 1999.

14. Ng, S. S. W., Tsao, M. S., Chow, S., and Hedley, D. W. Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. Cancer Res., *60:* 5451–5455, 2000.

15. Brognard, J., Clark, A. S., Ni, Y., and Dennis, P. A. 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, 2001.

16. Trayner, I. D., Bustorff, T., Etches, A. E., Mufti, G. J., Foss, Y., and Farzaneth, F. Changes in antigen expression on differentiating HL60 cells treated with dimethylsulfoxide, all-*trans* retinoic acid, α 1,25-dihydroxyvitamin D_3, or 12-*O*-tetradecanoyl phorbol-13-acetate. Leuk. Res., *27:* 537–547, 1998.

17. Bang, O-S., Park, E. K., Yang, S-I., Lee, S-R., Franke, T. F., and Kang, S-S. Overexpression of Akt inhibits NGF-induced growth arrest and neuronal differentiation of PC12 cells. J. Cell Sci., *114:* 81–88, 2001.

18. Klippel, A., Escobedo, J. A., Hirano, M., and Williams, L. T. The interaction of small domains between the subunits of phosphatidylinositol 3-kinase determines enzyme activity. Mol. Cell. Biol., *14:* 2675–2685, 1994.

19. Simpson, L. and Parsons, R. PTEN: life as a tumor suppressor. Exp. Cell Res., *264:* 29–41, 2001.

20. Zheng, W. H., Kar, S., and Quirion, R. Insulin-like growth factor-1-induced phosphorylation of the forkhead family transcription factor FKHRL1 is mediated by Akt kinase in PC12 cells. J. Biol. Chem., *275:* 39152–39158, 2000.

21. Li, Y. M., Schacher, D. H., Liu, Q., Arkins, S., Rebeiz, N., McCusker, R. H., Jr., Dantzer, R., and Kelley, K. W. Regulation of myeloid growth and differentiation by the insulin-like growth factor I receptor. Endocrinology, *138:* 362–368, 1997.

22. Grimberg, A. and Cohen, P. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. J. Cell. Physiol., *183:* 1–9, 2000.

23. Ogawa, M., Nishiura, T., Oritani, K., Yoshida, H., Yoshimura, M., Okajima, Y., Ishikawa, J., Hashimoto, K., Matsumura, I., Tomiyama, Y., and Matsuzawa, Y. Cytokines prevent dexamethasone-induced apoptosis via the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways in a new multiple myeloma cell line. Cancer Res., *60:* 4262–4269, 2000.

24. Vorwerk, P., Wex, H., Hohmann, B., Mohnike, K., Schmidt, U., and Mittler, U. Expression of the components of the IGF signalling system in childhood acute lymphoblastic leukaemia. Mol. Pathol., *55:* 40–45, 2002.

25. Huynh, H., Chow, P. K. W., Ooi, L. L. P., and Soo, K-C. A possible role for insulin-like growth factor-binding protein-3 autocrine/paracrine loops in controlling hepatocellular carcinoma cell proliferation. Cell Growth & Differ., *13:* 115–122, 2002.

26. Rajah, R., Lee, K-W., and Cohen, P. Insulin-like growth factor binding protein-3 mediates tumor necrosis factor-α-induced apoptosis: role of Bcl-2 phosphorylation. Cell Growth & Differ., *13:* 163–171, 2002.

27. Harada, H., Andersen, J. S., Mann, M., Terada, N., and Korsmeyer, S. J. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc. Natl. Acad. Sci. USA, *98:* 9666–9670, 2001.

28. Li, H-L., Davis, W., and Puré, E. Suboptimal cross-linking of antigen receptor induces Syk-dependent activation of p70S6 kinase through protein kinase C and phosphoinositol 3-kinase. J. Biol. Chem., *274:* 9812–9820, 1999.

29. Yamada, K. M. and Araki, M. Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. J. Cell Sci., *114:* 2375–2382, 2001.

30. Dahia, P. L., Aguiar, R. C., Alberta, J., Kum, J. B., Caron, S., Sill, H., Marsh, D. J., Ritz, J., Freedman, A., Stiles, C., and Eng, C. PTEN is inversely correlated with the cell survival factor Akt/PKB and is inactivated via multiple mechanisms in haematological malignancies. Hum. Mol. Genet., *8:* 185–193, 1999.

31. Aggerholm, A., Grønbæk, K., Guldberg, P., and Hokland, P. Mutational analysis of the tumour suppressor gene MMAC1/PTEN in malignant myeloid disorder. Eur. J. Haematol., *65:* 109–113, 2000.

32. Cuevas, B. D., Lu, Y., Mao, M., Zhang, J., LaPushin, R., Siminovitch, K., and Mills, G. B. Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase. J. Biol. Chem., *276:* 27455–27461, 2001.

33. Chan, T. O., Rodeck, U., Chan, A. M., Kimmelman, A. C., Rittenhouse, S. E., Panayotou, G., and Tsichlis, P. N. Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit. Cancer Cell, *1:* 181–191, 2002.

34. Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. Apoptosis: a link between cancer genetics and chemotherapy. Cell, *108:* 153–164, 2002.

35. Plo, I., Bettaïeb, A., Payrastre, B., Mansat-De Mas, V., Bordier, C., Rousse, A., Kowalski-Chauvel, A., Laurent, G., and Lautier, D. The phosphoinositide 3-kinase/Akt pathway is activated by daunorubicin in human acute myeloid leukaemia cell lines. FEBS Lett., *452:* 150–154, 1999.

36. Tallman, M. S., Andersen, J. W., Schiffer, C. A., Appelbaum, F. R., Feusner, J. H., Ogden, A., Shepherd, L., Willman, C., Bloomfield, C. D., Rowe, J. M., and Wiernik, P. H. All-*trans*-retinoic acid in acute promyelocytic leukemia. N. Engl. J. Med., *337:* 1021–1028, 1997.

37. Tari, A. M., Lim, S. J., Hung, M. C., Esteva, F. J., and Lopez-Berestein, G. Her2/neu induces all-*trans* retinoic acid (ATRA) resistance in breast cancer cells. Oncogene, *21:* 5224–5232, 2002.

38. Martelli, A. M., Bortul, R., Fackelmaye, F., Tazzari, P. L., Bareggi, R., Narducci, P., and Zweyer, M. Biochemical and morphological characterization of the nuclear matrix from apoptotic HL60 cells. J. Cell. Biochem., *72:* 35–46, 1999.

39. Martelli, A. M., Bortul, R., Bareggi, R., Grill, V., Narducci, P., and Zweyer, M. 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, 1999.

40. Martelli, A. M., Bortul, R., Bareggi, R., Tabellini, G., Grill, V., Baldini, G., and Narducci, P. 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, 1999.

41. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods, *139:* 271–279, 1991.

42. Ferrando-May, E., Cordes, V., Biller-Ckorvic, I., Mirkovic, J., Görlich, D., and Nicotera, P. Caspase mediate nucleoporin cleavage, but not early redistribution of nuclear transport factors and modulation of nuclear permeability in apoptosis. Cell Death & Differ., *8:* 495–505, 2001.

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