

Inhibition of Phosphatidylinositol 3'-Kinase/AKT Signaling Promotes Apoptosis of Primary Effusion Lymphoma Cells

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Abstract Purpose: Phosphatidylinositol 3'-kinase (PI3'-kinase) can be activated by the K1 protein of Kaposi sarcoma – associated herpes virus (KSHV). However, the role of PI3'-kinase in KSHV-associated primary effusion lymphoma (PEL) is not known. To assess this, we studied survival and apoptosis in PEL cell lines following inhibition of PI3'-kinase.

Experimental Design: Constitutive activation of several targets of PI3-kinase and apoptotic proteins were determined by Western blot analysis using specific antibodies. We used LY294002 to block PI3'-kinase/AKT activation and assess apoptosis by flow cytometric analysis.

Results: Blocking PI3'-kinase induced apoptosis in PEL cells, including BC1, BC3, BCBL1, and HBL6, whereas BCP1 was refractory to LY294002-induced apoptosis. LY294002-induced apoptosis did not seem to involve Fas/Fas-L but had an additive effect to CH11-mediated apoptosis. We also show that AKT/PKB is constitutively activated in all PELs and treatment with LY294002 causes complete dephosphorylation in all cell lines except BCP1 where a residual AKT phosphorylation remained after 24 hours of treatment. FKHR and GSK3 were also constitutively phosphorylated in PELs and treatment with LY294002 caused their dephosphorylation. Although inhibition of PI3'-kinase induced cleavage of BID in all cell lines, cytochrome *c* was released from the mitochondria and caspase-9 and caspase-3 were activated in LY294002-induced apoptotic BC1 but not in resistant BCP1. Similarly, XIAP, a target of AKT, was down-regulated after LY294002 treatment only in sensitive PEL cells.

Conclusions: Our data show that the PI3'-kinase pathway plays a major role in survival of PEL cells and suggest that this cascade may be a promising target for therapeutic intervention in primary effusion lymphomas.

Primary effusion lymphoma (PEL) is a subtype of non-Hodgkin's B-cell lymphoma that mainly presents in patients with advanced AIDS but is sometimes also found in HIV-negative individuals (1, 2). PEL grows as a lymphomatous effusion in body cavities and is always associated with Kaposi sarcoma-associated herpes virus (KSHV/HHV8). Most cases also show dual infection with EBV (EBV/HHV4; ref. 3). Pleural and abdominal effusions from patients with AIDS-PEL contain a number of cytokines (4, 5), which serve as autocrine growth factors for PELs. Interleukin 10 (IL-10) has been reported to serve as an autocrine growth factor for AIDS-related B-cell lymphoma (6). Recently, it has also been shown that PEL cells use viral IL-6 and IL-10 in an autocrine fashion for their

survival and proliferation (4). Most PELs become resistant against the conventional chemotherapeutic agents.

KSHV protein K1 has been shown to regulate a number of survival proteins. Studies have shown that K1 transgenic mice have constitutive activation of nuclear factor κ B and Oct-2 as well as activation of Src-kinase Lyn (7). Furthermore, K1 protein activates phosphatidylinositol 3'-kinase (PI3'-kinase/AKT) signaling pathway in B lymphocytes (8). AKT has been shown a target of a number of other transforming viral proteins including SV40 large and small T antigens, LMP1 and LMP2A (9–13). G protein-coupled receptor (vGPCR) of KSHV has been shown to transform cells by targeting AKT kinase (14–17). A recent study by Cannon and Cesarman has shown that vGPCR induces activation of AP1 and cAMP-responsive element-binding protein in PEL cells (18). Furthermore, KSHV infection in PEL leads to the activation of nuclear factor κ B, which plays a vital role in the survival of these cells (19).

Considerable evidence shows a role of PI3'-kinase/AKT signaling in oncogenic transformation and cancer progression (20). Deregulated PI3'-kinase signaling also provides attractive targets for therapy (21, 22). AKT prevents apoptosis by generating antiapoptotic signals through phosphorylation of Bad, GSK3, and caspase-9 and activation of transcriptional factors such as Forkhead (FOXO1) and nuclear factor κ B (23–27). These functions of PI3'-kinase make it a potential

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target for chemotherapeutic agents. Indeed, PI3'-kinase pathway inhibitors are already in early clinical trials.

In this study, we show for the first time that the PI3'-kinase/AKT pathway is constitutively activated in several PEL cell lines. Inhibition of PI3'-kinase activity by LY294002 not only caused dephosphorylation of basal levels of AKT, GSK3, and FKHR but also induced apoptosis in most PEL cell lines. Apoptosis occurred through release of cytochrome *c* from the mitochondria, activation of caspase-9 and caspase-3, and down-regulation of the inhibitor of apoptosis, XIAP.

Materials and Methods

Cell culture. Human primary effusion lymphoma cell lines established from lymphomatous effusion from patients with body cavity-based lymphomas, BC1, BC3, BCBL1, BCP1, and HBL6 were used. Cells were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. All PEL cell lines are infected with HHV8 and BC1 and HBL6 are also infected with EBV. These cell lines have been thoroughly characterized for immunophenotypic expression and genetic lesions (3–5).

Reagents and antibodies. LY294002 and anti-caspase-9 were obtained from Calbiochem (San Diego, CA). The anti-phospho-AKT, anti-phospho-GSK3 α/β , anti-phospho-FKHRL1, anti-cleaved caspase-3, and anti-BID antibodies were purchased from Cell Signaling Technologies (Beverly, MA). PTEN antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti- β -actin was purchased from Abcam (Cambridge, United Kingdom). Anti-cytochrome *c* and anti-XIAP antibodies were obtained from R&D Systems (Minneapolis, MN). Anti-caspase-3 was purchased from BD Pharmingen (San Diego, CA). The anti-poly(ADP-ribose) polymerase antibody was obtained from Zymed Lab (San Francisco, CA). Anti-Fas CH11 and ZB4 antibodies were purchased from MBL (Watertown, MA).

Apoptosis. PEL cell lines were treated with LY294002 as described in figure legends. Cells were harvested and the percentage apoptosis was measured by flow cytometry after staining with fluorescein-conjugated Annexin V and propidium iodide (PI; Molecular Probes, Eugene, OR) as described previously (28). We scored viable cells as those that are negative for Annexin V and PI. Percentage of apoptosis was calculated from the reduction of the number of viable cells between treated and untreated samples. The amount of necrotic cells (Annexin V negative, PI positive) was always minimal.

Cell lysis and immunoblotting. After treatment in various conditions, cells were collected by centrifugation at 1,000 rpm and lysed in phosphorylation lysis buffer (0.5–1.0% Triton X-100 or 1% digitonin, 150 mmol/L NaCl, 1 mmol/L EDTA, 200 μ mol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 1.5 mmol/L magnesium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μ g/mL aprotinin; ref. 29). Protein concentrations were assessed by the Bradford assay. Equal amount of proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon-PVDF; Millipore, Bedford, MA). Immunoblotting was done with different antibodies and developed using Enhanced Chemiluminescence Plus (Amersham, Buckinghamshire, United Kingdom).

Assay for cytochrome *c* release. Release of cytochrome *c* from mitochondria was assayed as described earlier (30). Briefly, cell pellets were resuspended in 5 volumes of a hypotonic buffer [20 mmol/L HEPES/KOH (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 20 μ g/mL leupeptin, 10 μ g/mL aprotinin, and 250 mmol/L sucrose] and incubated for 15 minutes on ice. Cells were homogenized by 15 to 20 passages through a 22-gauge needle, 1.5 in. long. The lysates were centrifuged at 1,000 \times g for 5 minutes at 4°C to pellet nuclei and unbroken cells. Supernatants were collected and centrifuge at 12,000 \times g for 15 minutes. The resulting

mitochondrial pellets were suspended in lysis buffer. Supernatants were transferred to new tubes and centrifuged again at 12,000 \times g for 15 minutes. These resulting supernatants represent cytosolic fractions. Twenty μ g of proteins from the cytosolic fraction of each sample were analyzed by immunoblotting using an anti-cytochrome *c* antibody.

Results

Inhibition of phosphatidylinositol 3'-kinase induces apoptosis in primary effusion lymphoma cells. The PI3'-kinase pathway has been implicated in the growth and survival of a range of cell types (31), but its effects on PEL cells have not been analyzed in detail. We sought to determine whether the inhibition of PI3'-kinase by its specific inhibitor, LY294002, caused apoptosis in PEL cells. LY294002 is a synthetic flavinoid that acts as a potent, competitive, reversible inhibitor of the ATP-binding site of class I PI3'-kinase (32). BC1 and BCP1 cells were treated with and without LY294002 for 24 hours and apoptosis was measured by Annexin V/PI dual staining. As shown in Fig. 1A, treatment with LY294002 of BC1 cells resulted in apoptosis (43.5 \pm 9%), whereas BCP1 showed no notable fraction of apoptotic cells (5 \pm 1%). We expanded the analysis to other PEL cell lines, including BC3 (62.7 \pm 2.4% apoptotic cells), BCBL1 (75 \pm 5.2%), and HBL6 (36 \pm 4.7%; Fig. 1B). A dose-response analysis revealed an increase of apoptotic cells beginning at a concentration of 10 μ mol/L with a maximum at 100 μ mol/L (Fig. 1C). Because nonspecific and toxic effects cannot be ruled out at 100 μ mol/L, we chose a working concentration of 50 μ mol/L, which has previously been shown specific for inhibition of PI3'-kinase (33). Figure 1D shows the kinetics LY294002-induced apoptosis in BC1 and BCP1 cells. Only BC1 showed a time-dependent apoptosis, whereas no appreciable amount of apoptosis occurred in BCP1. These data indicate that inhibition of PI3'-kinase results the induction of apoptosis in most PEL cells.

Effect of the agonistic (CH11) and antagonistic (ZB4) Fas antibodies on LY294002-induced apoptosis. Because it has been shown that the PI3'-kinase pathway can negatively modulate Fas ligand expression (26, 34), we investigated whether inhibition of PI3'-kinase involved Fas-mediated apoptosis in PEL. The addition of both LY294002 at 25 μ mol/L and CH11 at 50 ng/mL enhanced apoptosis compared with that obtained with either compound alone (Fig. 2A). The data also suggested that the combination of these two compounds had an additive rather than a synergistic effect.

We also studied the effect of the antagonistic Fas antibody (ZB4) on LY294002-induced apoptosis in PEL cell lines. BC1 cells were pretreated with 500 ng/mL of ZB4 or medium alone for 2 hours followed by treatment with 50 μ mol/L LY294002 for 24 hours and apoptosis was determined using Annexin V/PI dual staining. As a control for the activity of ZB4, we included 24 hours of treatment with CH11 (100 ng/mL). As shown in Fig. 2B, ZB4 notably reduced CH11-induced apoptosis but it exerted no effect on LY294002-induced apoptosis. Similar results were obtained using BCBL1 cells (data not shown). These results suggest that PI3'-kinase-mediated inhibition of apoptosis is independent of Fas/Fas-L in PEL cells.

Constitutive activation of PKB/AKT signaling pathways in primary effusion lymphoma cells. Activation of the PI3'-kinase has been studied in growth factor-independent cell lines

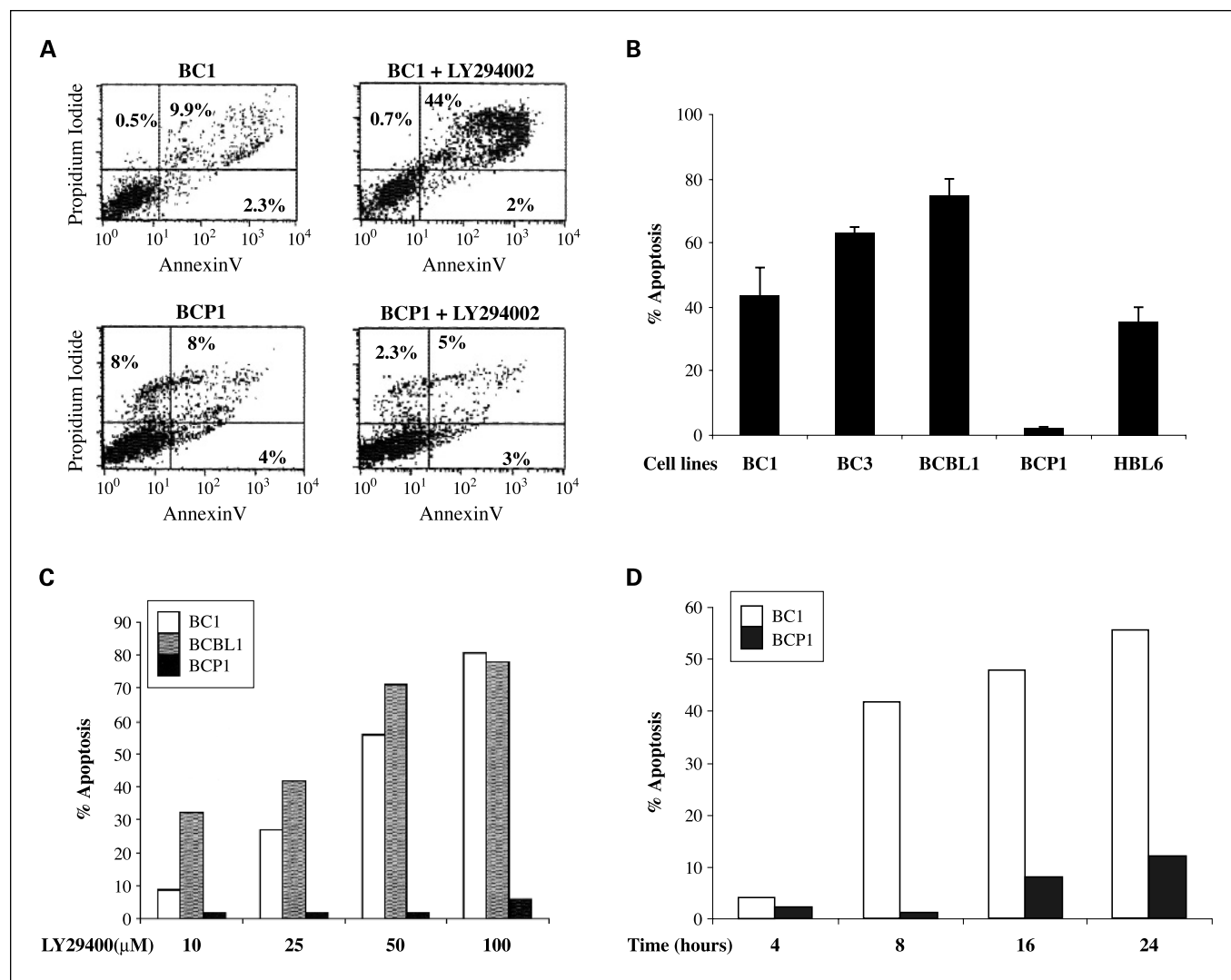


Fig. 1. LY294002-induced apoptosis in primary effusion lymphoma cells. *A*, BC1 and BCP1 cells were treated with and without 50 μmol/L LY294002 for 24 hours. Cells were subsequently stained with fluorescein-conjugated Annexin V and PI and analyzed by flow cytometry. *B*, columns, mean of three independent experiments; bars, ± SD. *C*, dose response of LY294002-induced apoptosis. As indicated, BC1, BCBL1, and BCP1 cells were treated with 10, 25, 50, and 100 μmol/L LY294002 for 24 hours and apoptosis was determined by Annexin V and PI staining. *D*, kinetics of LY294002-induced apoptosis when BC1 and BCP1 cells were treated with 50 μmol/L LY294002 for 4, 8, 16, and 24 hours.

through the phosphorylation of its downstream target, the serine-threonine kinase AKT (Ser⁴⁷³). By using an antibody that recognizes AKT at Ser⁴⁷³, we sought to determine the constitutive activation status of AKT in PEL cell lines as well as to determine whether inhibition of PI3'-kinase by LY294002 abrogates phosphorylation of AKT. PEL cell lines were treated in the presence and absence of LY294002 for various time periods as indicated, cells were lysed, and proteins were analyzed by Western blot. As shown in Fig. 3A, a, AKT is constitutively phosphorylated in the LY294002-sensitive BC1 cell line as well as in the resistant BCP1. Treatment with LY294002 dephosphorylated AKT completely in BC1 cells within 4 hours. Although LY294002 treatment reduced AKT phosphorylation in BCP1, residual levels remained even after 24 hours of treatment. Constitutive phosphorylation of AKT/PKB was also found in BCBL1 and HBL-6, which was completely dephosphorylated by LY294002 (data not shown).

The forkhead family of transcription factors has been reported as a downstream target of AKT, mediating apoptosis in other system (35). Active FKHR transcription factors promote transcription of genes involved in cell cycle arrest and apoptosis (36). One mechanism by which AKT promotes cell survival is by phosphorylating FKHR transcription factors, which inactivates them and prevents apoptosis (34). We thus studied the level of phosphorylation of FKHR/FOXO1 in LY294002-treated and untreated PEL cell lines by Western blotting. As shown in Fig. 3A, b, constitutive phosphorylation of FKHR was seen in BC1 and BCP1 cells and this phosphorylation was inhibited by treatment with the PI3' kinase inhibitor LY294002.

We next determined the activation of GSK3 in PEL cells, which has been recently reported a target of PI3'-kinase/AKT and is involved in promotion of cell survival (24). All PEL cell lines showed constitutive phosphorylation of GSK3 and dephosphorylation in the presence of LY294002 (Fig. 3A, c).

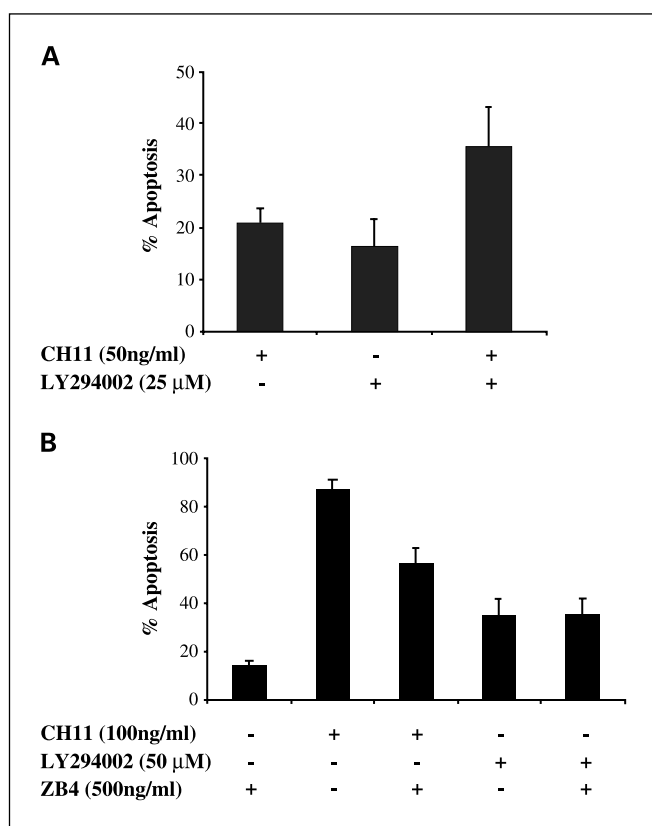


Fig. 2. LY294002-induced apoptosis does not involve the Fas/Fas ligand system but has additive effects to Fas-mediated apoptosis. *A*, BC1 cells were treated with either LY294002 at 25 μmol/L or the Fas agonistic CH11 antibody at 50 ng or both compounds simultaneously. After 24 hours, apoptosis was determined by Annexin V and PI staining. Column, mean of three independent experiments; bars, ±SD. *B*, effect of antagonistic Fas antibody (ZB4) on CH11- and LY294002-induced apoptosis. BC1 cells were pretreated for 2 hours with 500 ng/mL of ZB4 or medium alone and subsequently incubated with 50 μmol/L LY294002 or 100 ng/mL of CH11 for 24 hours. Columns, mean of three independent experiments in which apoptosis was estimated by Annexin V/PI dual staining; bars, ±SD.

Because we have used LY294402 as a major tool to show the role of PI3'-kinase signaling in PEL, we tested an unrelated signaling cascade, p38/mitogen-activated protein kinase by Western blotting to confirm specificity. As shown in Fig. 3B, BC1 and BCP1 cells treated with LY294002 showed phosphorylated p38 and the level of p38 activation was not affected by treatment, indicating specificity of PI3'-kinase inhibition.

Because lipid phosphatases have been shown to negatively regulate PI3'-kinase activity (37), we determined the expression of PTEN in PEL cell lines. As shown in Fig. 4, PTEN expression was detected in BC1, BCBL1, BCP1, and HBL6 but not in BC3 by Western blot. Because constitutive activation of AKT and LY294002-induced apoptosis apparently do not correlate with PTEN expression in PEL, the PI3'-kinase activity may not be related to PTEN loss in this system. Our results, however, do not rule out the possibility of a decreased PTEN activity in these cells.

Effects of the inhibition of phosphatidylinositol 3'-kinase/AKT signaling at the mitochondrial level in primary effusion lymphoma cells. The intrinsic apoptotic signaling cascade starts with truncation of BID that translocates to the mitochondrial membrane allowing activation of proapoptotic proteins and

release of cytochrome *c*. Therefore, we sought to determine whether inhibition of PI3'-kinase signaling induces BID cleavage in PEL cells. LY294002 treatment for 24 hours resulted in truncation of BID in BCBL1, BC1, and BCP1 cells (Fig. 5A) as inferred by the decreased intensity of the full-length BID band.

We then studied cytochrome *c* release from the mitochondria in cells treated for 24 hours with LY294002. Cytosolic-specific, mitochondria-free lysates were prepared as described in Materials and Methods. Cytochrome *c* was released to the cytosol after LY294002 treatment in BCBL1 and BC1 but not in BCP1 cells (Fig. 5B). These results suggest that BCP1 cells were able to resist PI3'-kinase/AKT inhibition-mediated apoptosis by short circuiting the intermediary signaling between BID and cytochrome *c* release.

Further downstream in the apoptotic pathway, we investigated whether inhibition of PI3'-kinase activates caspase-9 and caspase-3 and promotes cleavage of poly(ADP-ribose) polymerase. Figure 5C shows that LY294002 treatment results in the activation of caspase-9 and caspase-3 and cleavage of poly(ADP-ribose) polymerase in BC1 cells but not in BCP1 cells. These results are consistent with the data on cytochrome *c* release and indicate that activation of effector caspases participate in LY294002-induced apoptosis in PEL cells.

Down-regulation of XIAP in LY294002-induced apoptotic primary effusion lymphoma cells. XIAP is a physiologic substrate of AKT that is stabilized to inhibit programmed cell death and has a direct effect on caspase-3 and caspase-9 (38). To determine whether XIAP plays a role in protecting PEL cells from

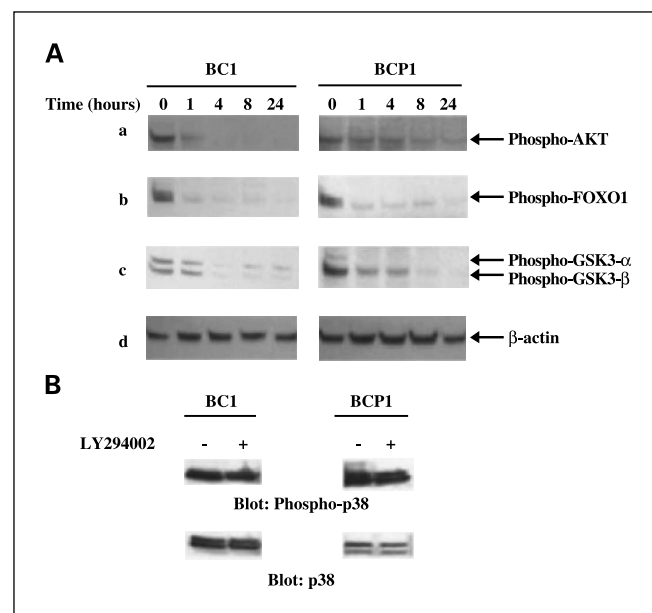


Fig. 3. Inhibition of PI3'/kinase/AKT signaling pathway during LY294002-induced apoptosis. *A*, BC1 and BCP1 cells were treated with and without 50 μmol/L LY294002 for the indicated time periods. After cell lysis, equal amounts of proteins were separated by SDS-PAGE, transferred to immobilon membrane, and immunoblotted with antibodies against (a) the phosphorylated/activated form of AKT on Ser⁴⁷³, (b) phosphorylated FOXO1; (c) phosphorylated form of GSK3α/β and (d) blots were stripped and reprobed with an antibody against actin. *B*, effect of PI3'/kinase inhibition on an unrelated mitogen-activated protein kinase/p38 signaling. BC1 and BCP1 were treated with 50 μmol/L LY294002 for 24 hours and assessed by immunoblot for phosphorylated and total level of p38.

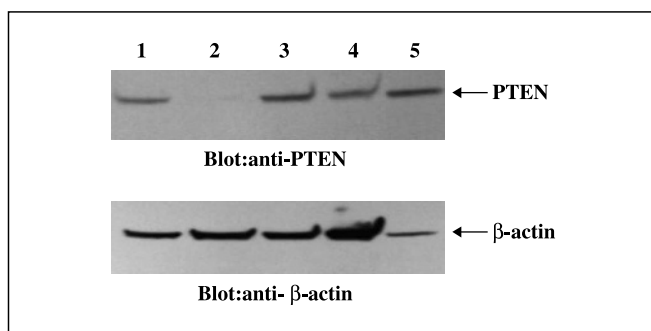


Fig. 4. Expression of inositol phosphatase PTEN in PEL cell lines. Lysates from (lane 1) BC1, (lane 2) BC3, (lane 3) BCBL1, (lane 4) BCP1, and (lane 5) HBL6 were analyzed with the indicated antibodies.

LY294002-induced apoptosis, BC1, BCP1, and BCBL1 cells were treated with and without LY294002. As shown in Fig. 6, expression of XIAP was significantly decreased in sensitive BC1 and BCBL1 cells after LY294002 treatment, whereas no effect was seen in the resistant BCP1 cell line. This data suggest that XIAP is an important survival molecule that mediates AKT-induced cell survival in PEL cells.

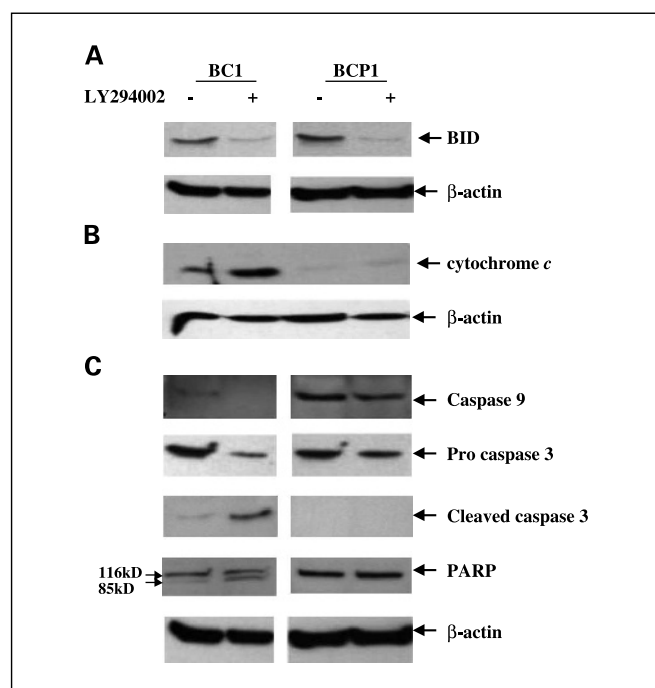


Fig. 5. LY294002-induced cleavage of BID, release of cytochrome *c* from mitochondria, and activation of caspase cascade in PEL cells. **A**, LY294002-induced cleavage of BID. BC1 and BCP1 cells were treated with and without 50 $\mu\text{mol/L}$ LY294002 for 24 hours. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to immobilized membrane, and immunoblotted with antibodies against BID (*top*). Blots were stripped and reprobbed with an antibody against actin (*bottom*). **B**, LY294002-induced cytochrome *c* release from mitochondria. BC1 and BCP1 cells were treated with and without 50 $\mu\text{mol/L}$ LY294002. Cytosolic fractions were prepared as described in Materials and Methods and immunoblotted with the corresponding antibodies. **C**, activation of caspase-9 and caspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP) induced by LY294002 treatment in PEL cells. BC1 and BCP1 cells were treated with and without 50 $\mu\text{mol/L}$ LY294002 for 24 hours. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with antibodies against caspase-9, procaspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase, and actin.

Discussion

PEL is a very aggressive and fatal type of cancer. These cells produce a variety of autocrine inflammatory cytokines and growth factors, including vIL6, vIL10, and VGF1, which provides them with cytoprotection against conventional chemotherapeutic agents (4–6). Constitutively activated signaling pathways are a common finding in hematologic malignancies (39–41). The amplification or up-regulation of PI3'-kinase/PKB-AKT signal transduction has been shown in the development of a variety of cancers (42, 43). Thus, targeting and down-regulating PI3'-kinase or AKT activity might contribute to cancer therapy.

In the present study, we provide evidence that constitutive activation of PI3'-kinase-PKB/AKT signaling pathway plays a critical role in regulating the growth and survival of PEL cells. Moreover, we showed that inhibition of PI3'-kinase pathway markedly induced apoptosis in all PEL cell lines studied except BCP1 (Fig. 1). We found that PI3'-kinase is frequently activated, as confirmed by the detection of constitutive phosphorylation of different substrates downstream of PI3'-kinase, including AKT, FKHR, and GSK3 in all PEL cells tested (Fig. 3). However, cell lines differed in the consequent effect on cytochrome *c* release from the mitochondria (Fig. 5).

The oncogenic role of the deregulated PI3'-kinase pathway is probably related to its simultaneous actions on growth and survival. Different mechanisms of PI3'-kinase deregulation and activation have been reported in different systems. Amplification of the p110 subunit of PI3'-kinase is observed in ovarian cancer (44) and this catalytic subunit found in a chicken tumor virus mediates its transforming effects through AKT (45). AKT is overexpressed in ovarian and pancreatic carcinomas (42, 43). In addition, inactivating mutations in the tumor suppressor gene *PTEN*, a negative regulator of PI3'-kinase activity, can induce uncontrolled AKT activity (37). We did not find any correlation between loss of *PTEN* and activation of AKT in PEL cell lines (Fig. 4), indicating that additional mechanisms downstream of *PTEN* contribute to the constitutively active PI3'-kinase pathway in PEL.

A recent study has shown that growth factor deprivation induces proteolytic cleavage of the proapoptotic Bcl-2 family member BID to yield its active truncated form, tBID (46).

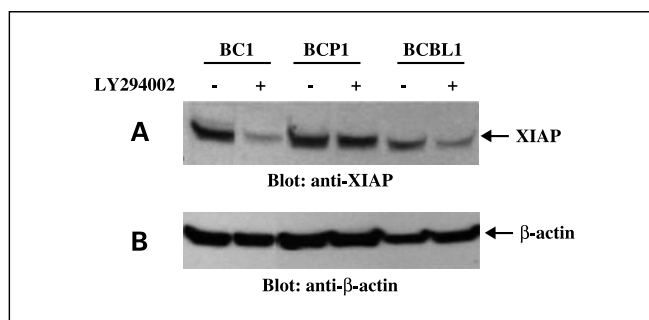


Fig. 6. LY294002-induced down-regulation of XIAP expression. BC1, BCP1, and BCBL1 cells were treated with and without 50 $\mu\text{mol/L}$ LY294002 for 24 hours. Cells were lysed and equal amounts of proteins were separated on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with (A) antibody against XIAP. Blots were stripped and reprobbed with an antibody against actin for equal loading (B).

However, activated AKT inhibited mitochondrial cytochrome *c* release and apoptosis following BID cleavage. In concordance with this, our data shows that inhibition of the PI3'-kinase/AKT pathway induced cleavage of BID in PELs, but cytochrome *c* was released from the mitochondria only in those cells (BC1) that had AKT completely dephosphorylated and not in cells with residual AKT phosphorylation (BCP1; Fig. 5B). Alternatively, the release of cytochrome *c* in BCP1 through BID activation was abrogated by an antiapoptotic mechanism at the mitochondrial level. Recently, it has been shown that AKT inhibits apoptosis downstream of BID cleavage involving hexokinases (46). AKT has also been shown to accumulate in the mitochondrial matrix and membrane after activation of PI3'-kinase (47). Moreover, the residual amount of phosphor-

ylated AKT in BCP1 suggests that AKT may protect XIAP from degradation in response to PI3'-kinase inhibition.

Together, our results establish that the PI3'-kinase/AKT pathway is constitutively activated in human PEL cell lines. Inhibition of PI3'-kinase leads to apoptosis in most PELs through release of cytochrome *c* from the mitochondria and activation of downstream caspases. These studies may have important implications for future preclinical and clinical studies in PEL. Indeed, they may pave the way for investigations aimed at determining the usefulness of a novel strategy for treating PEL with inhibitors of the PI3'/AKT pathway, alone or in combination with other agents. Further animal and preclinical studies are needed to validate the data presented here, which has greater effect with recent identification of small molecular inhibitors.

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