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Constitutively active Akt1 protects HL60 leukemia cells from TRAIL-induced apoptosis through a mechanism involving NF-κB activation and cFLIP$_L$ up-regulation

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TRAIL is a member of the tumor necrosis factor superfamily which induces apoptosis in cancer but not in normal cells. Akt1 promotes cell survival and blocks apoptosis. The scope of this paper was to investigate whether a HL60 human leukemia cell clone (named AR) with constitutively active Akt1 was resistant to TRAIL. We found that parental (PT) HL60 cells were very sensitive to a 6 h incubation in the presence of TRAIL and died by apoptosis. In contrast, AR cells were resistant to TRAIL concentrations as high as 2 μg/ml for 24 h. Two pharmacological inhibitors of PI3K, Ly294002 and wortmannin, restored TRAIL sensitivity of AR cells. AR cells stably overexpressing PTEN had lower Akt1 activity and were sensitive to TRAIL. Conversely, PT cells stably overexpressing a constitutive active form of Akt1 became TRAIL resistant. TRAIL activated caspase-8 but not caspase-9 or -10 in HL60 cells. We did not observe a protective effect of Bcl-X$_L$ or Bcl-2 against the cytotoxic activity of TRAIL, even though TRAIL induced cleavage of BID. There was a close correlation between TRAIL sensitivity and intranuclear presence of the p50 subunit of NF-κB. Higher levels of the FLICE inhibitory protein, cFLIP$_L$, were observed in TRAIL-resistant cells. Both the cell permeable NF-κB inhibitor SN50 and cycloheximide lowered cFLIP$_L$ expression and restored sentivity of AR cells to TRAIL. Our results suggest that Akt1 may be an important regulator of TRAIL sensitivity in HL60 cells through the activation of NF-κB and up-regulation of cFLIP$_L$ synthesis

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Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is one of the latest members of the TNF superfamily known to induce apoptosis in a wide variety of cancer cells.[1–4] The messenger RNA for TRAIL and its receptors are nonetheless expressed in a wide range of normal tissues[5] so that a TRAIL physiological role has yet to be envisioned, even though TRAIL shows inhibitory effects on erythroblasts,[6] T lymphocytes,[7] and hepatocytes.[8] TRAIL induces apoptosis by binding DR4 (TRAIL-R1) and DR5 (TRAIL-R2/KILLER/TRICK2) receptors. DR4 and DR5 are type I transmembrane proteins that transduce the death signal via their 80-amino acid intracellular death domains (DDs), which recruit adaptor proteins.[9] TRAIL receptors are ubiquitously expressed in cancer cells.[10] Depending on the cell type being investigated, binding of TRAIL to DR4 and DR5 leads to activation of caspase-8 or -10, that in turn, activates downstream effector caspases, caspase-3 and -7 with an ensuing irreversible commitment to apoptotic cell death.[11] In some cell types, activation of caspase-8 by TRAIL may also cleave BID (a Bcl-2 inhibitory protein) whose cleavage product triggers mitochondrial depolarization and subsequent release of cytochrome c from mitochondria and activation of caspase-9.[12] Two additional TRAIL receptors, TRAIL-R3/TRID/DcR1 and TRAIL-R4/TRUNDD/DcR2, lack completely (DcR1) or partially (DcR2) a DD and therefore cannot induce a proapoptotic signal. Thus, they are considered 'decoy receptors', competing with DR4 and DR5 for TRAIL binding.[13,14] Recently, based on *in vitro* experiments, some TRAIL-resistant cell lines have been discovered. The reason for TRAIL resistance is unknown, but it is not regulated exclusively by the expression of DcR1 and DcR2. Instead, it seems that intracellular inhibitors acting downstream of TRAIL receptors are responsible for resistance.[12] One of the most effective antiapoptotic survival pathways in mammals is constituted of phosphoinositide 3-kinase (PI3K)/Akt.[15] To date, three different Akt isoforms have been identified: Akt1, -2, and -3.[16,17] Akt (also referred to as PKB, protein kinase B) is a serine/threonine protein kinase activated in response to many stimuli including growth factors such as insulin, insulin-like growth factor-1, epidermal growth factor, or cytokines such as interleukin-3, interleukin-6, macrophage colony-stimulating factor.[18,19] These agonists stimulate PI3K which synthesizes 3-phosphoinositides such as phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3]. PtdIns(3,4,5)P_3 activates the phosphoinositide-dependent protein kinase PDK-1 which is recruited at the plasma membrane.[16,17] PDK-1 phosphorylates Akt1 on Thr 308. It is commonly thought that PtdIns (3,4,5)P_3 causes a conformational change in Akt with an ensuing increase of the accessibility to the phosphorylation site. However, for a full activation of Akt1, phosphorylation of Ser 473 is also required. The kinase responsible for this phosphorylation is yet to be identified, but it might be PDK-1 or PDK-2, ILK (integrin-linked kinase) or Akt itself.[16,17]

Evidence suggests that Akt inhibits apoptosis through multiple mechanisms. These include direct phosphorylation and inactivation of proapoptotic proteins such as Bad or procaspase-9, increased expression of antiapoptotic proteins (cIAP1, cIAP2, TRAF-1, TRAF-2, cFLIP, Bcl-2, Mcl-1, A1/Bfl-1), and down-regulation of proapoptotic proteins such as FAS-L, IGFBP1, Bim.[16,17] Very recently, some articles have demonstrated that, in cell lines derived from prostatic cancers, Akt1 protects from apoptosis induced by TRAIL (eg Refs 12, 20 and 21).

The purpose of this study was to investigate the intracellular mechanisms by which an apoptosis-resistant (named AR) cell clone of HL60 human leukemia cells is insensitive to TRAIL. We found that these cells have a constitutively active Akt1. While in TRAIL-sensitive parental (PT) HL60 cells, TRAIL activates caspase-8, in HL60AR cells this activation did not occur. Treatment of HL60AR cells with two pharmacological inhibitors of PI3K, wortmannin and Ly294002, restored sensitivity to TRAIL. If HL60AR cells were transfected with the cDNA coding for PTEN, they reacquired TRAIL sensitivity. Con-

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versely, forced overexpression of a constitutively active Akt1 rendered HL60PT cells resistant to TRAIL. Expression of the long variant of the caspase-8 homologue FLICE inhibitory protein (cFLIP$_L$, a potent negative regulator of death receptor-induced apoptosis) was up-regulated in HL60AR as compared to PT cells. Both the cell-permeable NF-κB inhibitor, SN50, and cycloheximide reversed TRAIL resistance in HL60AR cells and down-regulated the levels of cFLIP$_L$. These data suggest that elevated Akt1 activity protects HL60AR cells from TRAIL-induced apoptosis through a mechanism which involves NK-κB and cFLIP$_L$.

Materials and methods

Materials

G418, etoposide, bovine serum albumin (BSA), normal goat serum (NGS), normal goat IgG, protein A-Agarose, peroxidase-conjugated and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Sigma (St. Louis, MO, USA). Histone H2B, the COMPLETE Protease Inhibitor Cocktail, and the Lumi-Light^{Plus} enhanced chemiluminescence (ECL) detection kit were from Roche Applied Science (Milan, Italy). Constitutively active Akt1 murine cDNA (myc-tagged, cloned in pUSEamp) and PTEN assay kit were from Upstate Biotechnology (Lake Placid, NY, USA). Phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P_2], Ly 294002, wortmannin, the caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) cell-permeable inhibitors, cycloheximide (CHX) and human recombinant TRAIL were from Calbiochem (La Jolla, CA, USA). PtdIns(3,4,5)P_3 was from Alexis Biochemical (Laufelfingen, Switzerland). The SN50 peptide was from Biomol Research Laboratories (Plymouth Meeting, PA, USA). The cDNA encoding full-length human PTEN (cloned in pcDNA3) was a kind gift from Dr S Marmiroli (Istituto di Citomorfologia Normale e Patologica del CNR, Bologna, Italy). The ApoAlert Caspase-8 colorimetric assay kit was from BD Biosciences Clontech (Palo Alto, CA, USA). The Intraprep kit for cytoplasmic staining was purchased from Coulter Immunology (Hialeah, FL, USA). The Protein Assay kit (detergent compatible) was from Bio-Rad (Hercules, CA, USA). cAMP-dependent protein kinase inhibitor peptide was from Bachem (Bubendorf, Switzerland). P-81 paper was from Whatman (Maidstone, UK).

Source of antibodies

The following primary antibodies were used in this study: from Upstate Biotechnology: rabbit polyclonals to Akt1, cytochrome c, mouse monoclonals to human c-myc (clone 9E10) and caspase-9; from Santa Cruz Biotechnology (Santa Cruz, CA): mouse monoclonals to NF-κB p50 subunit and FLICE-inhibitory protein (cFLIP$_{S/L}$); from Cell Signaling Technology (Beverly, MA, USA): mouse monoclonals to PTEN and BID, rabbit polyclonals to caspase-10, Thr 308 phosphorylated Akt1 (p-Akt1), Ser 473 p-Akt1, Bcl-2, Bcl-X$_L$; from Sigma: mouse monoclonal to β-tubulin, rabbit polyclonal to procaspase-8; from R&D Systems (Oxon, UK): goat polyclonals to extracellular domain of human DR4, DR5, DcR1, DcR2; from Molecular Probes (Leiden, The Netherlands): rabbit polyclonal to cytochrome oxidase subunit II (COX II).

Cell culture and differentiation

The HL60 cell line (HL60PT, for parental), obtained from the American Type Culture Collection (CCL 240), was routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum at an optimal cell density of 3 to 8 $\times$ 10^5 cells/ml.

Induction and detection of apoptosis

Sixteen hours after medium change, HL60 cells in exponential growing phase were treated with TRAIL for 6 h or 24 h at 37°C. In some experiments, prior to apoptotic induction, cells had been pre-incubated for 24 h with wortmannin (0.1 μM) or Ly294002 (5 μM). For detection of apoptosis, samples containing 2 to 5 $\times$ 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 sub-diploid DNA content was calculated as described by Nicoletti *et al*[22] using an Epics XL flow cytometer with the appropriate software (Coulter Immunology).

Flow cytometric detection of TRAIL receptors

For surface staining the antibodies (concentration 50 μg/ml) were incubated at 4°C for 12 h in a final volume of 50 μl with 3 $\times$ 10^5 cells. After two washes in PBS cells were incubated with FITC-conjugated anti-goat IgG. For analysis of intracytoplasmic receptors, we employed a saponin-based technique.[23] Briefly, cells were fixed with Reagent 1 of the Intraprep kit, following the manufacturer's instructions. Then, cells were permeabilized with saponin-based Reagent 2 and incubated at 4°C for 12 h with the antibodies (concentration as above). Samples were then incubated with FITC-conjugated anti-goat IgG. Negative controls were run with normal goat IgG plus FITC-conjugated anti-goat IgG. All samples were analyzed by Epics XL flow cytometer equipped with dedicated software.

In vivo labeling of polyphosphoinositides, lipid extraction and thin layer chromatography

Cells were incubated with phosphate-free RPMI 1640/0.5% BSA for 15 min, then exposed to the same medium plus carrier-free ^{32}P ortophosphate (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 $CaCl_2$ and 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 10 μg of a 1:1:1 mixture of phosphatidylinositol/phosphatidylinositol (4) phosphate/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 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 unlabelled standards PtdIns (4,5)P_2, and PtdIns (3,4,5)P_3. Spots of interest were scraped and counted by liquid scintillation.

Expression of wild-type PTEN and constitutively active (myristolated) Akt1

Transfection of HL60 cells was performed by electroporation with a Bio-Rad Gene Pulser apparatus. Twenty μg 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.

Measurement of caspase-8 activity

This was performed using a colorimetric assay according to the manufacturer's instructions. Briefly, samples (100 μg of protein) were incubated with the caspase-8 colorimetric substrate (IETD-pNA) at 37°C for 2 h. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

Preparations of mitochondria and cytosol

Cell pellets were resuspended with 5 vol ice-cold buffer A (20 mM Hepes-KOH, pH 7.5, 0.1% BSA, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml of both aprotinin and pepstatin A) containing 250 mM sucrose. After swelling on ice for 5 min, the cells were homogenized with 20 strokes of a number 22 Kontes Dounce homogenizer with the B pestle (Kontes Glass, Vineland, NJ, USA) and the homogenates were centrifuged at 750 *g* for 15 min at 4°C. Supernatants were then pelleted at 10 000 *g* for 15 min at 4°C. Resultant pellets containing mitochondria were resuspended in cold buffer A. Supernatants were further cleared at 20 000 *g* for 30 min at 4°C and used as cytosol. For Western blot analysis, equal amounts of mitochondrial and cytosol were separated by gel electrophoresis.

Preparations of cell homogenates and Western blot analysis

Cells were washed twice in PBS containing the COMPLETE Protease Inhibitor Cocktail supplemented with 1.0 mM Na_3VO_4 and 20 nM okadaic acid. Cells 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 ECL method. To ensure equal loading, blots were always first probed with an antibody to β-tubulin, then stripped and re-probed.

Preparation of cell homogenates for immunoprecipitation

To obtain homogenates, cells were resuspended in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, supplemented with the protease and phosphatase inhibitor cocktail and homogenized by 30 passages through a 25-gauge needle. Cell homogenates (1 ml, containing 500 μg of protein) were pre-cleared by adding 5 μg of normal rabbit IgG and 10 μg of 50% Protein A-Agarose, followed by incubation for 1 h at 4°C and centrifugation at 12 000 *g* for 10 min at 4°C. Then, the samples were incubated for 2 h at 4°C under constant agitation with 5 μg of a polyclonal antibody to Akt1. Ten μg of 50% Protein A-Agarose was then added and incubation proceeded for 1 h at 4°C under constant agitation.

Akt1 activity assay

The immunoprecipitates were washed twice in lysis buffer, once in distilled water and twice in the Akt1 kinase buffer (20 mM Hepes-NaOH, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT) as previously reported.[24] Assays (100 μl) contained 20 mM Hepes-NaOH, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 1 μM cAMP-dependent protein kinase inhibitor peptide, 5 μg histone H2B as exogenous substrate, 2 μM ATP, 10 μCi γ-^{32}P ATP (3000 Ci/mmole). Samples were incubated for 30 min at 30°C and the reaction was then stopped by spotting 80 μl on to P-81 filter papers and immersing in 1% (v/v) orthophosphoric acid. The papers were washed several times, rinsed in ethanol, air-dried, and the radioactivity was determined by scintillation counting. Background values, obtained by samples in which the anti-Akt1 antibody was replaced by normal rabbit IgG, were subtracted from all values.

PTEN activity assay

The assay was performed using the manufacturer's recommendations.

Immunofluorescent staining

Cells in PBS were plated on to 0.1% poly-L-lysine-coated glass slides and adhesion was allowed to proceed for 30 min at room temperature. Samples were fixed in methanol for 10 min at −20°C. After several washes with PBS, nonspecific binding of antibodies was blocked by a 30 min incubation at 37°C with PBS, 2% BSA, 5% NGS. Slides were then incubated for 3 h at 37°C with the antibody to NF-κB p50 subunit diluted 1:50 in PBS, 2% BSA. Slides were then washed three times in PBS and reacted with FITC-conjugated anti-rabbit IgG, diluted 1:200 in PBS, 2% BSA, 5% NGS for 1 h at 37°C. Samples were subsequently washed three times in PBS, stained for DNA with 0.5 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) in PBS and mounted in 20 mM Tris-HCl, pH 8.2, 90% glycerol, containing 2.3% of the antifading agent 1,4-diazobicyclo-[2.2.2]-octane. Slides were observed and photographed using a Zeiss Axiophot epifluorescence microscope.

Results

Akt1 activity in HL60 cells and its inhibition by PI3K inhibitors

During our previous studies on apoptosis,[25,26] we selected a cell population which was resistant to drugs, such as campto-

thecin and etoposide, that we employed to induce apoptosis in HL60 cells. A clone was further isolated on soft agar. We named this clone HL60AR cells (for apoptosis resistant). As shown in Figure 1a, Western blotting analysis revealed that the amount of total Akt1 was similar in HL60PT and AR cells. Treatment of AR cells with two pharmacological inhibitors of PI3K, Ly294002 and wortmannin, did not lower the amount of total Akt1.

However, when the blots were probed with antibodies to either Thr 308 or Ser 473 p-Akt1, it was evident that HL60AR cells contained more p-Akt1 than HL60PT cells (Figure 1a). The levels of p-Akt1 forms of AR cells could be markedly reduced by a 24 h incubation in the presence of either Ly294002 or wortmannin (Figure 1a). Consistently, the *in vitro* kinase activity of Akt1 was higher in HL60AR when compared to PT cells. The Akt1 activity of HL60AR cells could be markedly inhibited by treatment with Ly294002 or wortmannin (Figure 1b).

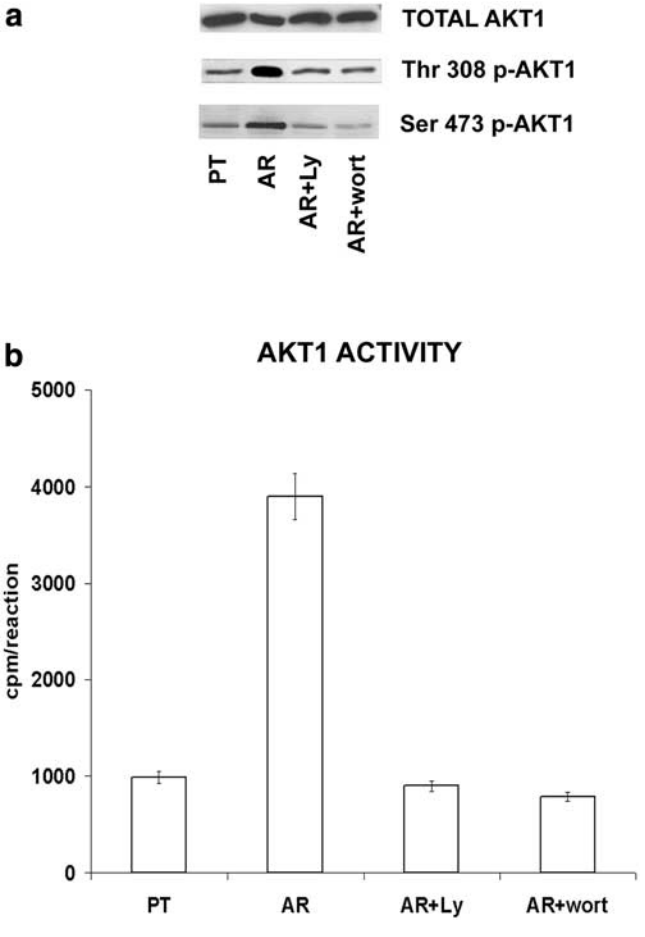

Figure 1 Akt1 activity in HL60PT or AR cells. Western blot analysis for expression of total Akt1 and p-Akt1 forms (a). For inhibition with Ly294002 (Ly, 5 μM) or wortmannin (wort, 0.1 μM) cells were incubated in the presence of the inhibitors for 24 h. 80 μg of protein was blotted to each lane. The blots are representative of at least three separate experiments. (b) *In vitro* Akt1 kinase activity. For inhibition with Ly294002 (Ly) or wortmannin (wort) cells were incubated in the presence of the inhibitors for 24 h. The results are the mean ± s.d. of three separate experiments.

TRAIL sensitivity of HL60 cells

As shown in Figure 2, TRAIL induced apoptosis in HL60PT cells. A 20 ng/ml concentration was capable of causing apoptotic cell death in 34% of cells after a 6 h incubation. A 500 ng/ml concentration killed 68% of cells. The efficacy of TRAIL did not rise significantly if higher concentrations (up to 2 μg/ml) were employed. In contrast, HL60AR cells were markedly resistant to TRAIL so that a 2 μg/ml concentration resulted in only about 14% of cells being apoptotic. Furthermore, AR cells were resistant to TRAIL even if the incubation was prolonged to 24 h, thus demonstrating that the apoptotic process was not simply delayed in these cells (data not shown).

If AR cells had been pre-treated with Ly294002 or wortmannin, they became much more sensitive to TRAIL. For example, whereas a 1 μg/ml TRAIL concentration killed only approximately 10% of AR cells, this percentage rose to about 50% after treatment with the inhibitors. Importantly, Ly294002 or wortmannin alone did not induce apoptosis in HL60AR cells (Figure 2).

Analysis of TRAIL receptors

In principle, the lower sensitivity to TRAIL displayed by AR cells could be due to an expression of TRAIL receptors different from PT cells. Therefore, we investigated this issue. As shown in Figure 3a, Western blotting analysis of DR4, DR5 and DcR2 showed that they were all expressed to the same extent in HL60PT and AR cells. We did not detect DcR1 receptor in our cells. Moreover, flow cytometric analysis allowed us also to conclude that the DR4 and DR5 receptors present at the cell surface did not vary between the two kinds of cells (Figure 3b). DcR2 receptor was detected only in permeabilized cells, suggesting it is located in the cytoplasm. However, in this case there were also no differences between PT and AR cells (Figure 3b).

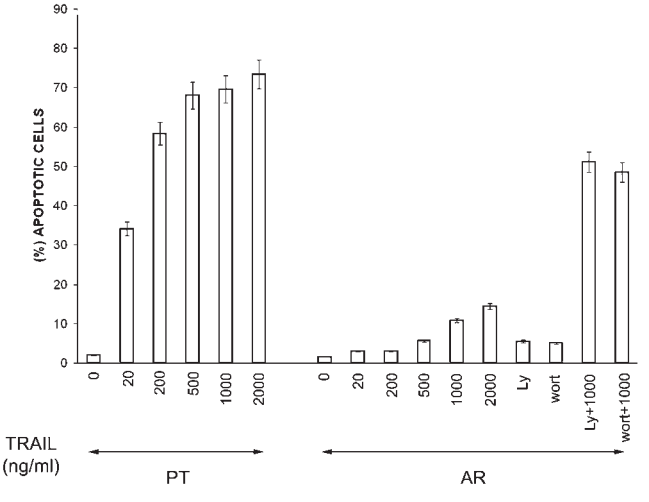

Figure 2 Effect of TRAIL on HL60PT or AR cells. Flow cytometric analysis for apoptosis in HL60 cells treated for 6 h with increasing concentrations of human recombinant TRAIL. For inhibition with Ly294002 (Ly) or wortmannin (wort) cells were incubated in the presence of the inhibitors for 24 h. The results are the mean ± s.d. of three separate experiments.

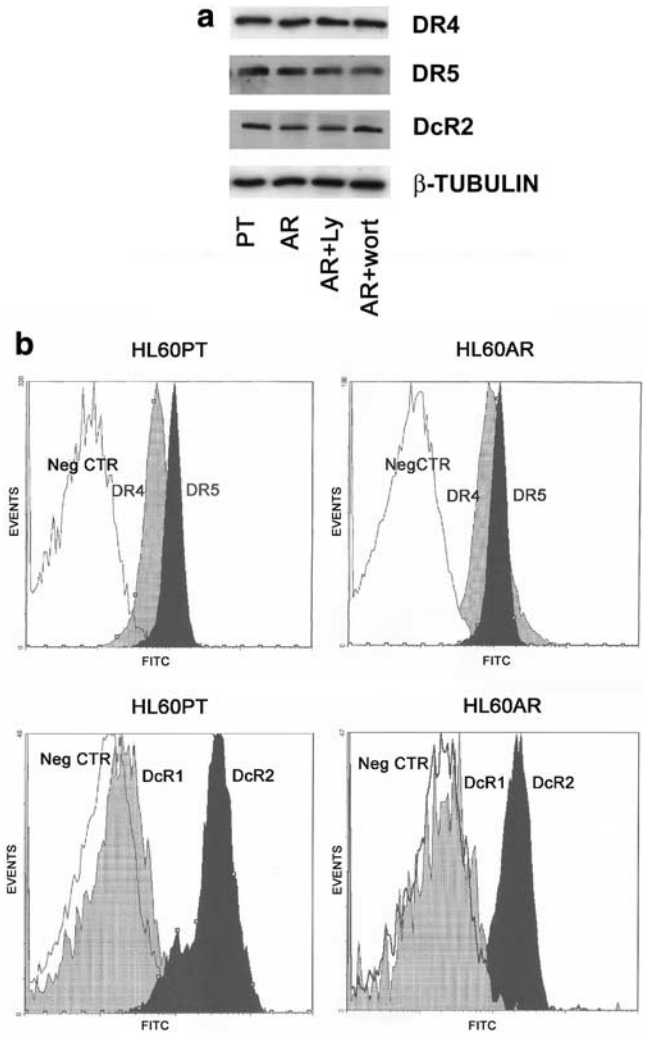

Figure 3 Expression of TRAIL receptors in HL60PT or AR cells. (A) Western blot analysis. For inhibition with Ly294002 (Ly) or wortmannin (wort) cells were incubated in the presence of the inhibitors for 24 h. 80 μg of protein was blotted to each lane. Equal protein loading was ensured by demonstration of uniform β-tubulin expression. The blots are representative of at least three separate experiments. (B) Flow cytometric analysis. NEG CTR: negative control (cells were incubated with normal goat IgG followed by FITC-conjugated anti-goat IgG). The histograms shown are representative of three separate experiments.

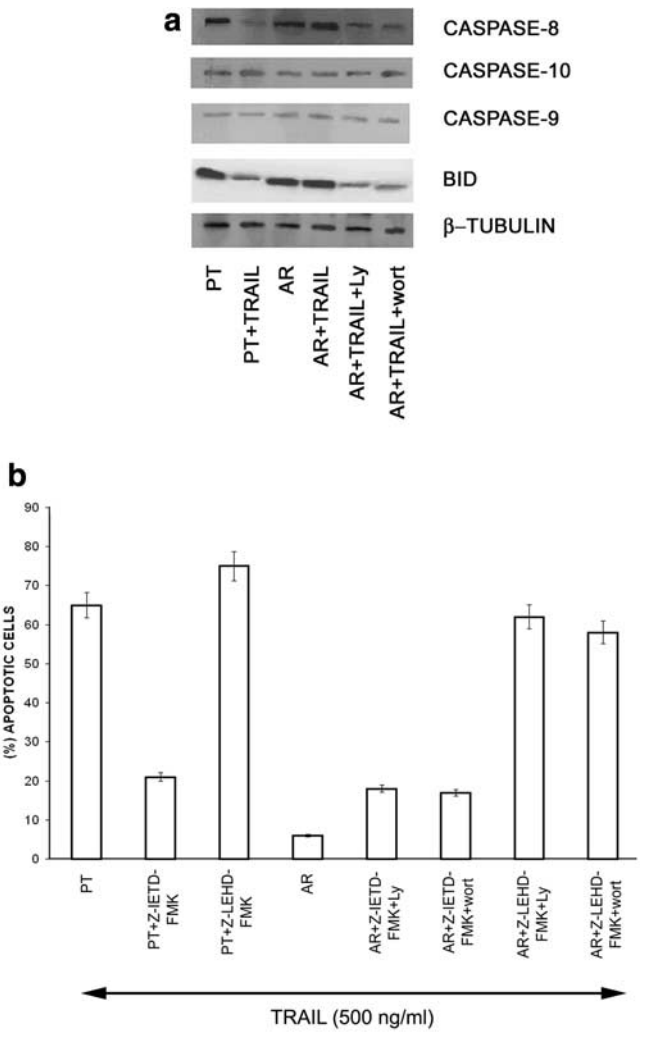

Figure 4 Western blot analysis for caspases and BID and modulation of apoptosis by caspase inhibitors in HL60PT or AR cells. (A) Western blot analysis. Cells were incubated for 6 h with 1 μg/ml TRAIL. For inhibition with Ly294002 (Ly) or wortmannin (wort) cells were pre-incubated in the presence of the inhibitors for 24 h. Note that the antibody to caspase-8 we employed in this study only recognizes the procaspase-8 banding at 55/54 kDa. 80 μg of protein was blotted to each lane. Equal protein loading was ensured by demonstration of uniform β-tubulin expression. The blots are representative of at least three separate experiments. (B) Flow cytometric analysis showing the effect of caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) inhibitors on TRAIL-induced apoptosis. Cells were pre-treated for 1 h with the inhibitors (used at 25 μM) before a 6 h incubation with TRAIL. For inhibition with Ly294002 (Ly) or wortmannin (wort) cells were pre-incubated in the presence of the inhibitors for 24 h. The results are the mean ± s.d. of three separate experiments.

Caspase activation by TRAIL

We next analyzed the caspase activation pattern in response to TRAIL. As shown in Figure 4a, incubation with TRAIL (1 μg/ml for 6 h) induced the cleavage of procaspase-8 in PT cells, but not in AR cells. However, if AR cells had been pre-treated with Ly294002 or wortmannin, TRAIL-dependent cleavage of procaspase-8 was detected by Western blot. In contrast, we never detected cleavage of procaspase-10 or procaspase-9 in HL60 cells, either PT and AR. BID was cleaved in HL60PT cells treated with TRAIL, whereas it was not in AR cells plus TRAIL. However, in AR cells pre-treated with Ly294002 or wortmannin TRAIL exposure resulted in BID cleavage.

As presented in Figure 4b, an inhibitor of caspase-8 markedly reduced apoptosis in PT cells treated with 500 ng/ml TRAIL. On the other hand, a caspase-9 inhibitor was ineffective. In AR cells pre-treated with Ly294002 or wortmannin, a caspase-8 inhibitor markedly reduced TRAIL-dependent apoptosis. In contrast, in AR cells pre-treated with Ly294002 or wortmannin an inhibitor of caspase-9 was still ineffective in preventing apoptosis.

Bcl-X_L and Bcl-2 expression and cytochrome c release in HL60PT and AR cells

Since previous results indicated that both Bcl-X_L and Bcl-2, when overexpressed in HL60 cells, could block TRAIL-induced apoptosis,[27,28] we analyzed the expression levels of

these two antiapoptotic proteins in HL60PT and AR cells. As shown in Figure 5a, Western blots showed that Bcl-X_L was indeed much more abundant in AR than in PT cells. However, treatment with PI3K inhibitors, Ly294002 and wortmannin, which restored TRAIL sensitivity of AR cells, did not lower the amount of Bcl-X_L present in these cells. As far as Bcl-2 was concerned, its expression was lower in AR than in PT cells (Figure 5b). As further evidence that TRAIL did not induce apoptosis through the mitochondrial pathway in HL60 cells, we analyzed cytochrome c release from mitochondria. As presented in Figure 5c, we never detected an increase in the amount of cytosolic cytochrome c in either PT cells treated with TRAIL or in AR cells pre-treated with Ly294002 followed by incubation with TRAIL, ie two conditions which resulted in a large number of HL60 cells undergoing apoptosis. In contrast, there was a massive release of cytochrome c from mitochondria in etoposide-exposed PT cells (Figure 5c), a treatment which resulted in about 50% of apoptotic cells (not shown). COX II served as a control to rule out gross mitochondrial contamination of the cytosolic extracts.[29]

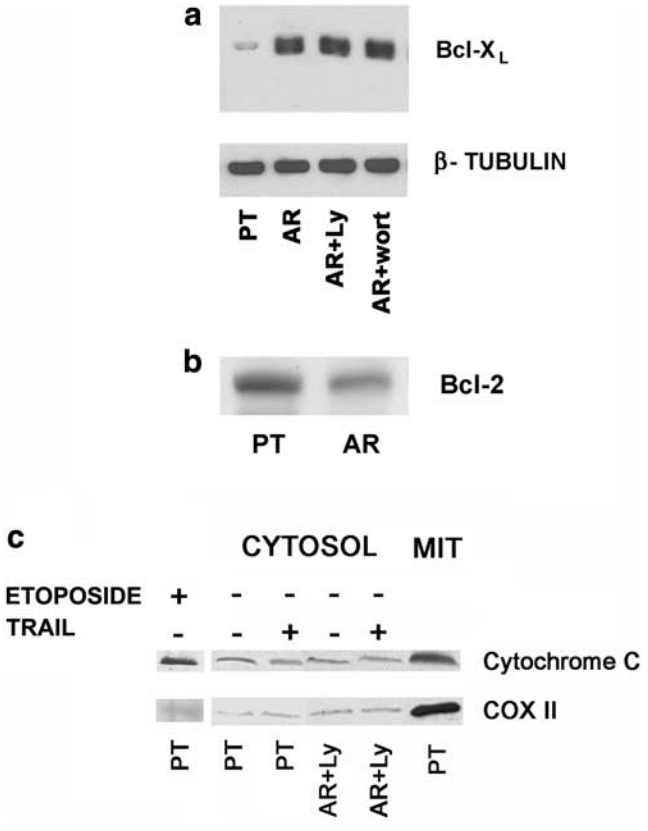

Figure 5 Bcl-X_L and Bcl-2 expression and cytochrome c release in HL60PT and AR cells. Western blot analysis for Bcl-XL (a) or Bcl-2 (b). 80 μg of protein was blotted to each lane. In (a) equal protein loading was ensured by demonstration of uniform β-tubulin expression. (C) Western blot analysis for cytochrome c and COX II in cytosol and mitochondria (MIT). Cell were treated with 500 ng/ml TRAIL or 5.0 μg/ml etoposide for 6 h. For inhibition with Ly294002 (Ly) cells were pre-incubated in the presence of the inhibitors for 24 h. 80 μg of protein was blotted to each lane. The blots are representative of at least three separate experiments.

Overexpression of PTEN restores TRAIL sensitivity in HL60AR cells

Since the dual phosphatase PTEN, by converting PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2, is a regulator of Akt1 activity,[30] we sought to examine the effects of human PTEN overexpression on TRAIL-induced apoptosis in HL60AR cells. Cells were transfected with either the empty vector or the full-length construct. As shown in Figure 6a, we selected three stably transfected clones that, by Western blotting, displayed enhanced expression of PTEN. In contrast, mock-transfected cells had PTEN levels comparable to untransfected HL60AR cells. We next assayed the levels of PtdIns(3,4,5)P_3 synthesized *in vivo* by these clones. As presented in Figure 6b, all three of the clones had PtdIns(3,4,5)P_3 levels markedly lower than untransfected HL60AR cells. Also, *in vitro* PTEN activity was increased in these clones (Figure 6c), whereas *in vitro* Akt1 kinase activity was decreased (Figure 6d). Cell clones overexpressing PTEN, but not mock-transfected cells, were sensitive to a 6 h treatment with 500 ng/ml TRAIL (Figure 5e).

Overexpression of constitutively active Akt1 renders HL60PT cells resistant to TRAIL

We next tried to determine whether or not overexpression of a constitutively active (myristolated) Akt1 could protect HL60PT cells from TRAIL-induced apoptosis. Also in this case, cells were transfected with either the empty vector or the full-length construct. In Figure 7a, we show that we selected three stably transfected cell clones overexpressing similar amounts of Akt1 protein. To reveal the bands, we used an antibody to the myc tag. Therefore, when we assayed untransfected and mock-transfected HL60PT cells, we did not detect any bands corresponding to Akt1. *In vitro* Akt1 kinase activity was increased in these clones (Figure 7b). If these clones were exposed for 6 h to 2 μg/ml TRAIL, only about 11–14% of cells underwent apoptotic cell death (Figure 7c).

Analysis of NF-κB immunofluorescent intracellular distribution

To exert its anti-apoptotic effect, Akt1 targets several downstream proteins.[16,17] We examined NF-κB transcription factor. When inactive, this protein resides as a complex in the cytoplasm. Following Akt1 activation, the p50 subunit of NF-κB migrates to the nucleus.[17] Therefore, we studied the intracellular distribution of the p50 NF-κB subunit by immunofluorescence staining. In HL60PT cells, this subunit was localized in the cytoplasm (Figure 8a, b). In contrast, in AR cells, it was present within the nucleus (Figure 8c, d). If AR cells were treated with Ly294002, the p50 NF-κB subunit subcellular distribution resembled that of PT cells (Figure 8e, f). Also if AR cells had been treated with SN50 (a synthetic peptide which inhibits intranuclear translocation and transcriptional activity of NF-κB, see Ref. 31) p50 NF-κB subunit was mainly distributed in the cytoplasm (Figure 8g, h). p50 NF-κB subunit was also cytoplasmic in cell clone 4H1 (which overexpresses PTEN, see Figure 8i, j), whereas it was nuclear in clone 12FC (which overexpresses constitutively active Akt1, see Figure 8k, l).

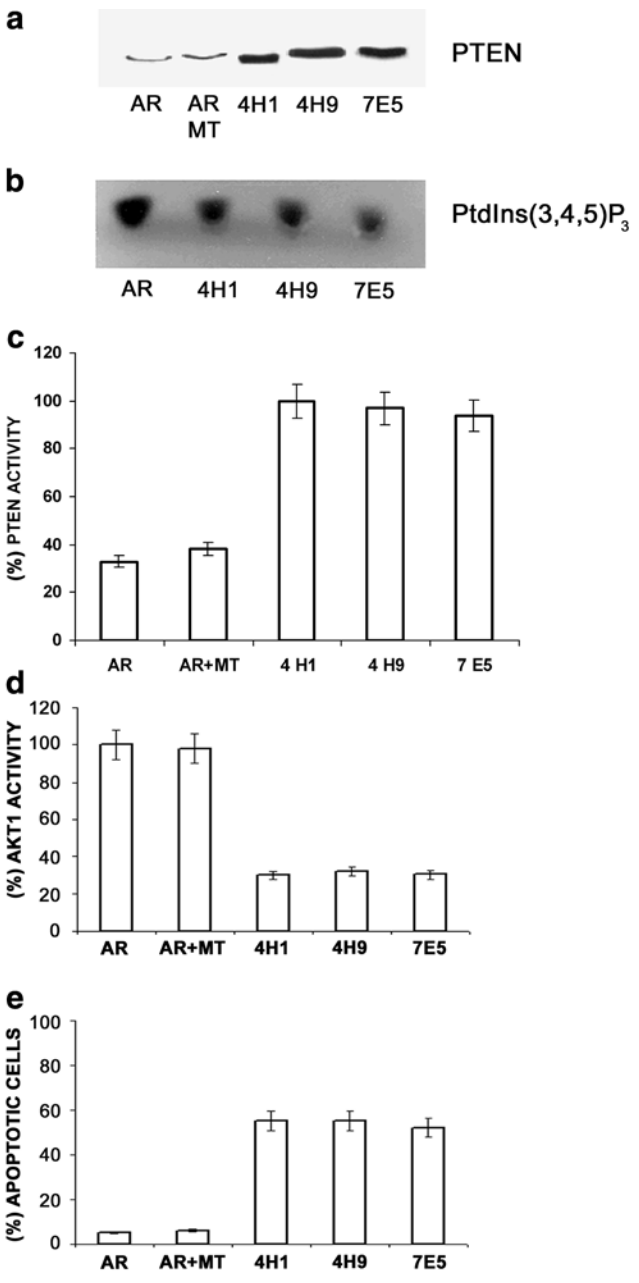

Figure 6 Overexpression of human PTEN sensitizes HL60AR cells to TRAIL. (A) Western blot analysis for PTEN expression in HL60AR, mock-transfected HL60AR cells (AR MT) and in three stably transfected clones. 80 μg of protein was blotted to each lane. The blot is representative of at least three separate experiments. (B) Levels of *in vivo* synthesized PtdIns (3,4,5)P_3 in HL60AR cells and in the three stably transfected clones overexpressing PTEN. A representative autoradiograph from three separate experiments is shown. In this experiment, incorporation was 1987 c.p.m. for AR cells, 604 c.p.m. for clone 4H1, 559 c.p.m. for clone 4H9 and 517 c.p.m. for clone 7E5. (C) *In vitro* PTEN activity in HL60AR, AR MT cells, and in three stably transfected clones; 100% of the activity was 257.1 ± 21.4 pmoles P released/reaction. (D) *In vitro* Akt1 kinase activity in HL60AR, AR MT cells, and in three stably transfected clones; 100% of the activity was 4087 ± 356 c.p.m. incorporated/reaction. (E) Flow cytometric analysis for TRAIL-induced apoptosis (500 ng/ml TRAIL for 6 h) in HL60AR, AR MT cells, and in three stably transfected clones. In c,d, e, the results are the mean ± s.d. of three separate experiments.

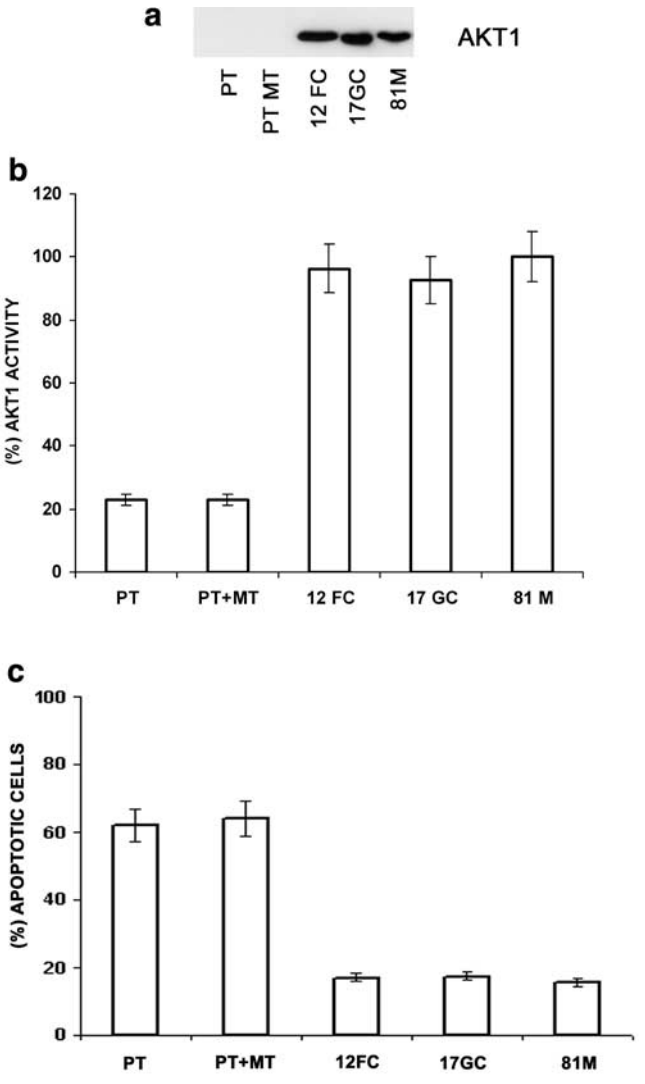

Figure 7 Overexpression of constitutively active Akt1 renders HL60PT cells resistant to TRAIL. (A) Western blot analysis for Akt1 expression in HL60PT, mock-transfected HL60PT cells (PT MT), and in three stably transfected clones. 80 μg of protein was blotted to each lane. Overexpressed Akt1 was revealed by means of a monoclonal antibody to the myc-tag. The blot is representative of at least three separate experiments. (B) *In vitro* Akt1 kinase activity in HL60PT, PT MT cells, and in three stably transfected clones; 100% of the activity was 4234 ± 378 c.p.m. incorporated/reaction. (C) Flow cytometric analysis of TRAIL-induced apoptosis (2 μg/ml TRAIL for 6 h) in HL60PT, PT MT cells, and in three stably transfected clones. In (B) and (C) the results are the mean ± s.d. of three separate experiments.

cFLIP and TRAIL receptor expression in relationship to Akt1 and NF-κB activity

Recent evidence has indicated that the degenerate caspase homologue cFLIP, whose expression is controlled by the PI3K/Akt axis, can inhibit TRAIL-dependent apoptosis in prostate tumor cells.[32] Moreover, it seems that in some cell lines, NF-κB plays a key role in regulating expression of cFLIP, which is inhibited by CHX.[33] Since in AR cells procaspase-8 was not processed to active caspase 8, this suggested that TRAIL resistance might arise from inhibition at the level of caspase-8 activation. We therefore decided to analyze cFLIP expression in HL60 cells. Western blotting analysis showed that $cFLIP_L$ was less abundant in PT than in AR cells (Figure

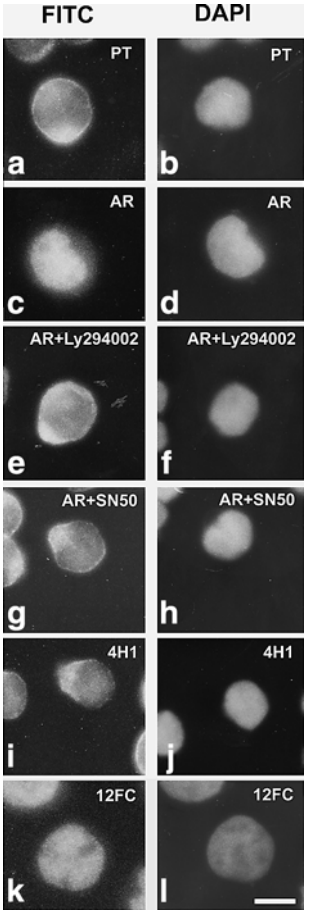

Figure 8 Immunofluorescent analysis for NF-κB subcellular distribution. HL60PT or AR cells, were fixed, permeabilized and immunostained by an antibody to the p50 subunit of the NF-κB transcription factor, that was then revealed by a FITC-conjugated secondary antibody. DNA was revealed by DAPI counterstaining. For treatment with Ly294002 cells were pre-incubated in the presence of the inhibitor for 24 h. In the case of SN50, the inhibitor (30 μg/ml) was present for 6 h. The pictures are representative of three separate experiments. Scale bar: 5 μM.

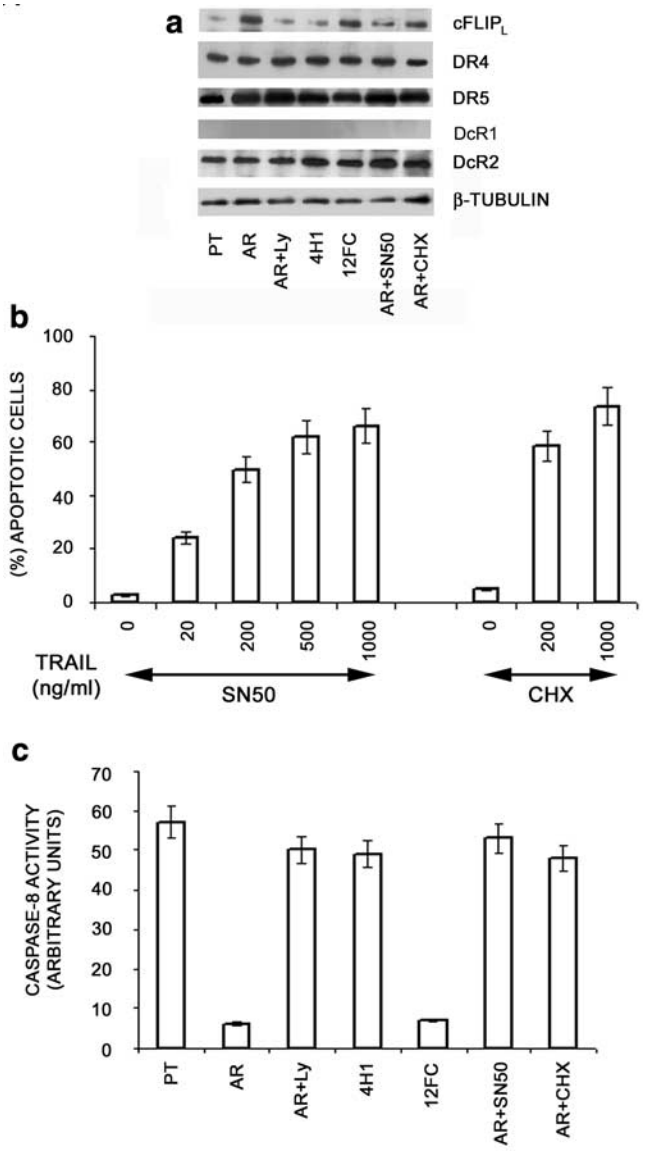

Figure 9 cFLIP$_L$ expression, TRAIL-induced apoptosis and caspase-8 activity correlate with NF-κB activity. (A) Western blot analysis for cFLIP$_L$, DR4, DR5 and DcR2 in HL60 PT and AR cells and cell clones 4H1 and 12FC. Equal protein loading was ensured by demonstration of uniform β-tubulin expression. The blot is representative of at least three separate experiments. (B) Flow cytometric analysis showing the effect of SN50 or CHX on TRAIL-induced apoptosis in HL60AR cells. Cells were incubated with TRAIL for 6 h after treatment with either SN50 or CHX. (C) Demonstration of caspase-8 activity in response to a 6 h incubation with 500 ng/ml TRAIL of HL60 PT and AR cells and cell clones 4H1 and 12FC. In (b) and (c) the results are the mean ± s.d. of three separate experiments. For treatment with Ly294002 cells were pre-incubated in the presence of the inhibitor for 24 h. In the case of SN50, the inhibitor (30 μg/ml) was present for 6 h. In the experiments with CHX, the drug was present for 6 h at 0.1 μg/ml.

9a). If AR cells had been exposed to Ly294002, the cFLIP$_L$ levels were similar to those of PT cells. The same was also true of clone 4H1, overexpressing PTEN, whereas clone 12FC (with a forced overexpression of constitutively active Akt1) had cFLIP$_L$ levels comparable to AR cells. Finally, treatment with either SN50 or CHX lowered the cFLIP$_L$ amount produced by AR cells. Although the antibody we employed recognizes both the large and small forms of cFLIP, we never detected the small form by Western blot. However, we detected its mRNA by RT-PCR, but the amount was similar in both PT and AR cells (not shown).

Evidence suggests that NF-κB transcription factor may protect cells from TRAIL-induced apoptosis by up-regulating the levels of the decoy receptor DcR1.[34] We therefore sought to determine whether or not there was a relationship between TRAIL receptors expression and Akt1 and/or NF-κB activity. However, as presented in Figure 9a, it was evident that DcR1 was not expressed after treatment of HL60 AR cells with Ly294002, SN50 or CHX, and in clones 4H1 or 12FC. Moreover, the levels of the three expressed receptors also did not change under the same conditions.

cFLIP$_L$ levels correlate with apoptosis and caspase-8 activity

As presented in Figure 9b, there was a close relationship between apoptosis and the amount of cFLIP$_L$ expressed by HL60AR cells. Treatment with either SN50 or CHX markedly increased the sensitivity of these cells to TRAIL. After exposure to 200 ng/ml TRAIL, caspase-8 activity was higher in PT than

AR cells, in which it could be raised by exposure to Ly294002. Caspase-8 activity was also high in clone 4H1 and in AR cells treated with either SN-50 or CHX (Figure 9c).

Discussion

In this paper, we have shown for the first time that Akt1 is a crucial mediator of TRAIL resistance in HL60 leukemia cells. Such a conclusion is based on several findings. *In vivo* phosphorylation and *in vitro* kinase activity were up-regulated in a clone of HL60 cells, named AR cells. Both phosphorylation and activity of Akt1 could be down-regulated by two unrelated pharmacological inhibitors of PI3K, Ly294002 and wortmannin. While HL60PT cells were highly sensitive to TRAIL treatment, HL60AR cells displayed a strong resistance so that a 6 h incubation in the presence of 2 μg/ml TRAIL induced apoptosis in only 14% of cells. The sensitivity to TRAIL of HL60AR cells did not increase even if the incubation was prolonged to 24 h. However, pre-incubation of HL60AR cells with Ly294002 or wortmannin concentrations that down-regulated Akt1 phosphorylation and activity restored sensitivity to TRAIL. To gain further evidence of the important role played by Akt1 in conferring TRAIL resistance to HL60AR cells we transfected these cells with PTEN. We selected three stably transfected clones in which PTEN amount and activity was higher than in AR cells. In these clones the amount of *in vivo* synthesized PtdIns(3,4,5)P_3 was lower than in AR cells. Also, Akt1 phosphorylation and *in vitro* kinase activity were down-regulated in the three clones. The three clones were sensitive to a TRAIL concentration (500 ng/ml) to which AR cells were resistant. Furthermore, we transfected PT cells with constitutively active Akt1 cDNA and we selected three stably transfected clones overexpressing Akt1. These clones had Akt1 kinase activity significantly higher than PT cells and were resistant up to 2 μg/ml TRAIL. Therefore, the results gained through a genetic approach also strongly supported the hypothesis that up-regulation of Akt1 is of primary importance for the TRAIL resistance observed in HL60AR cells.

We have ruled out the possibility that the TRAIL resistance of HL60AR cells was due to a different expression of TRAIL receptors when compared to PT cells. Indeed, Western blotting analysis showed that DR4, DR5 and DcR2 receptors were expressed to the same extent in the two types of cells. In agreement with the findings of others[27,28] we did not find DcR1 in HL60 cells. Also, flow cytometric analysis demonstrated that the receptor expression on the cell surface or in the cytoplasm did not vary between PT and AR cells.

TRAIL activated procaspase-8 but not procaspase-10 or -9 in HL60PT cells. This finding is consistent with the report of others,[11] who showed procaspase-8 to be the apical caspase of TRAIL-induced apoptosis in hematopoietic malignancies.[11,35,36] In contrast, recruitment of procaspase-10 to the TRAIL receptors DR4 and DR5 has been demonstrated in some solid tumors.[37,38] We did not detect cleavage of procaspase-8 in AR cells. However, if these cells had been exposed to PI3K inhibitors, procaspase-8 cleavage became evident. Also, the use of selective caspase inhibitors strongly suggested an involvement of caspase-8 but not of caspase-9 in TRAIL-mediated apoptotic death of HL60 cells, since Z-IETD-FMK, an inhibitor of caspase-8, blocked apoptosis of HL60PT cells, whereas Z-LEHD-FMK, an inhibitor of caspase-9, was ineffective. Consistent with these findings, AR cells, pre-treated with either Ly294002 or wortmannin, did not undergo apoptosis in the presence of TRAIL plus Z-IETD-FMK, while they did if Z-LEHD-FMK was employed together with TRAIL. Similar results were recently reported for K562 cells.[39]

However, we detected TRAIL-evoked BID cleavage in PT cells or in AR cells exposed to PI3K inhibitors. Interestingly, in agreement with the findings of others, we did not detect the cleavage products of BID,[28] even though the antibody we employed does. Moreover, we did not see release of cytochrome c from mitochondria in PT cells treated with TRAIL. Once released from mitochondria, cytochrome c in complex with apoptotic protease-activating factor 1 (Apaf-1) activates caspase-9, which in turn activates the executioner caspases.[40,41] In some types of cells, referred to as type II cells, caspase-8 activation leads, through the cleavage of BID, to the release of cytochrome c from mitochondria. On the other hand, cells of hematopoietic lineage, are considered to be type I cells, that is cells in which TRAIL does not activate the mitochondrial pathway of apoptosis.[11] Taken together, our results strongly suggest that the mitochondrial pathway, even though BID is cleaved, is not activated by TRAIL in HL60 cells. We are presently investigating why, despite the BID cleavage, there is no cytochrome c release from mitochondria.

Our findings suggest that neither Bcl-X_L nor Bcl-2 is capable of blocking TRAIL-induced apoptosis in the HL60 cells we employed. These findings are not consistent with data from others who showed that both of these proteins, when ectopically overexpressed, could block TRAIL-dependent apoptosis of HL60 cells by inhibiting activation of procaspase-8.[28] It should be emphasized, however, that the mechanism through which Bcl-2 and/or Bcl-X_L would inhibit receptor-mediated apoptosis is not clear. Both Bcl-2 and Bcl-X_L reside on the outer mitochondrial membrane, whereas caspase-8 is on the plasma membrane as a component of the TRAIL–receptor complex. At present, we have no explanation for the discrepancy between our findings and the results of others,[28] although we could hypothesize that Bcl-2 and/or Bcl-X_L are capable of blocking TRAIL-evoked apoptosis only if they are overexpressed and produced in an amount which is not physiological. Indeed, it might be that these proteins, when overexpressed, exist as soluble forms capable of blocking the activation of membrane-bound procaspase-8.

We have explored the possibility that Akt1 up-regulation observed in HL-60 AR cells exerts its antiapoptotic effect through the transcription factor NF-κB. Indeed, Akt phosphorylates and activates IKK-α, which in turns phosphorylates I-κB, thus promoting the nuclear translocation of NF-κB. NF-κB controls the transcription of genes fundamental for survival, such as cFLIP, cIAP1, cIAP2, TRAF-1 and TRAF-2.[17] The results of immunofluorescence analysis showed that the p50 subunit of NF-κB was localized in the cytoplasm of HL60PT cells, whereas in AR cells it was present in the nucleus. If AR cells were treated with Ly294002 the p50 subunit was located in the cytoplasm. This intracellular distribution of the p50 subunit was also seen when AR cells were treated with SN50, a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence of the p50 subunit. SN50 inhibits nuclear translocation and transcriptional activity of NF-κB.[41] Moreover, p50 subunit was cytoplasmic in clone 4H1 whereas it was intranuclear in clone 12FC.

HL60AR expressed levels of the caspase degenerate homologue cFLIP$_L$ higher than PT cells. cFLIP$_L$ expression was sensitive to treatment of AR cells with Ly294002, wortmannin, SN50 and CHX. Moreover, the amount of cFLIP$_L$ was lower in clone 4H1 and higher in clone 12FC. These findings suggest that in HL60 cells the expression of cFLIP$_L$ is regulated by Akt1 through a NF-κB-dependent mechanism. There was

a close relationship between the levels of $cFLIP_L$ expressed by HL60 cells and the activity of caspase-8. High levels of $cFLIP_L$ were coupled with a low caspase-8 activity and vice versa. Importantly, treatment with SN50 or CHX sensitized HL60AR cells to the proapoptotic action of TRAIL.

cFLIP is rapidly emerging as a key molecule which can block death receptor-mediated apoptosis. Indeed, this protein is capable of binding to the adapter protein FADD (Fas-associated death domain) which is a key component of the death-inducing signaling complex. However, cFLIP cannot be cleaved to an active caspase thus preventing the initiation of the death pathway. In some types of solid tumors it has been found that Akt regulates the expression of cFLIP.[32] There are several alternative spliced transcripts of cFLIP in mammalian cells, but only two can be detected at the protein level: the short form ($cFLIP_S$) and the long form ($cFLIP_L$).[42] Very recently, a higher constitutive expression of $cFLIP_L$ in B chronic lymphocytic leukemia cells was observed as compared to normal tonsillar B cells.[43] It is of great interest that B-chronic lymphocytic leukemia cells were relatively resistant to TRAIL-induced apoptosis. It should be recalled that in other cell types, such as HeLa cells, $cFLIP_S$ appears to be a major cellular inhibitor of TRAIL-induced apoptosis.[44]

Our findings also ruled out that NF-κB activation could lead to changes in the expression levels of TRAIL receptors. Indeed, there is a study in which an up-regulation of DcR1 was shown to be dependent on NF-κB activity and could make HeLa cells resistant to TRAIL.[34]

Overall, the data presented here indicate that in HL60AR cells TRAIL resistance is mediated by an activation of the Akt1/NF-κB pathway. So far, Akt1 has been linked to TRAIL resistance only in prostatic cancer cells,[12,20,21,45] but our results indicate that this serine/threonine protein kinase may also affect TRAIL sensitivity in neoplastic cells of hematopoietic lineage. Akt can be regarded as a powerful antiapoptotic oncoprotein and, as such, it is one of the most attractive kinase targets in cancer cells for the development of new pharmacological tools to sensitize cells to treatments inducing apoptosis. TRAIL may be a novel therapeutic agent for human neoplastic diseases, including those of hematopoietic origin.[27] In light of our findings, treatment with agents that down-regulate Akt activity should be useful in cases of TRAIL resistance due to an up-regulation of the PI3K/Akt pathway. Moreover, our data confirm the usefulness of pharmacological agents that down-regulate NF-κB activity to overcome resistance to TRAIL.[11,27,46]

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