

Resveratrol Induces Extensive Apoptosis by Depolarizing Mitochondrial Membranes and Activating Caspase-9 in Acute Lymphoblastic Leukemia Cells¹

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ABSTRACT

Resveratrol, a plant antibiotic, has been found to have anticancer activity and was recently reported to induce apoptosis in the myeloid leukemia line HL60 by the CD95-CD95 ligand pathway. However, many acute lymphoblastic leukemias (ALLs), particularly of B-lineage, are resistant to CD95-mediated apoptosis. Using leukemia lines derived from patients with pro-B t(4;11), pre-B, and T-cell ALL, we show in this report that resveratrol induces extensive apoptotic cell death not only in CD95-sensitive leukemia lines, but also in B-lineage leukemic cells that are resistant to CD95-signaling. Multiple dose treatments of the leukemic cells with 50 μM resveratrol resulted in $\geq 80\%$ cell death with no statistically significant cytotoxicity against normal peripheral blood mononuclear cells under identical conditions. Resveratrol treatment did not increase CD95 expression or trigger sensitivity to CD95-mediated apoptosis in the ALL lines. Inhibition of CD95-signaling with a CD95-specific antagonistic antibody indicated that CD95-CD95 ligand interactions were not involved in initiating resveratrol-induced apoptosis. However, in each ALL line, resveratrol induced progressive loss of mitochondrial membrane potential as measured by the dual emission pattern of the mitochondria-selective dye JC-1. The broad spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone failed to block the depolarization of mitochondrial membranes induced by resveratrol, further indicating that resveratrol action was independent of upstream caspase-8 activation via receptor ligation. However, increases in caspase-9 activity ranged from 4- to 9-fold in the eight cell lines after treatment with resveratrol. Taken together, these results point to a general mechanism of apoptosis induction by resveratrol in ALL cells that involves a mitochondria/caspase-9-specific pathway for the activation of the caspase cascade and is independent of CD95-signaling.

INTRODUCTION

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a member of the class of plant antibiotics known as phytoalexins and functions to protect the plant from fungal infection (1). This antifungal agent is present in the skin of grapes and in a narrow range of other spermatophytes. Resveratrol has received wide attention for its high concentration in red wine and for its proposed role in reducing the risk of coronary heart disease. Recently, Jang *et al.* (2) described the ability of resveratrol to inhibit events associated with the initiation, promotion, and progression of cancer. The cancer preventive activity of resveratrol was linked to its ability to eliminate free radicals and to reduce oxidative and mutagenic stress. Furthermore, this agent can inhibit directly enzymes associated with cell proliferation and DNA-replication, such as DNA polymerase (3) and ribonucleotide reductase (4). It was shown that resveratrol acted as a radical scavenger by interacting with and destroying the tyrosyl radical on the R2 subunit of ribonucleotide reductase, which is necessary for the activity of this enzyme.

CD95-mediated apoptosis is a key mechanism for the regulation of the immune system and has been implicated in the elimination of abnormally developed hematopoietic progenitor cells. Triggering of the CD95 pathway by CD95L³ induces the formation of the DISC, consisting of CD95, Fas-associated protein with death domain, and caspase-8 (FLICE/MACH; Refs. 5 and 6). Upon recruitment into the DISC, caspase-8 is activated by proteolytic cleavage, which then initiates the apoptotic cascade by cleavage of effector caspases, most notably caspase-3 (7–9). Activation of the caspase cascade results in numerous changes in the cell that define apoptosis, such as endonucleolytic cleavage of DNA, proteolytic cleavage of nuclear and cellular proteins, cell membrane blebbing, and production of apoptotic bodies (10). CD95-CD95L interactions have been implicated in the action of chemotherapeutic agents in some experimental systems, but not in others (11–13). However, it has recently been proposed that chemical-induced apoptosis is not mediated through activation of caspase-8, but rather involves the mitochondrial release of factors, such as cytochrome *c*, which in turn activate caspase-9 (14). In these experiments, the caspase activation pathways during receptor and chemical-mediated apoptosis were investigated using the broad spectrum caspase inhibitor Z-VAD-FMK. This inhibitor was capable of blocking disruption of mitochondrial membranes induced by receptor-mediated activation of caspase-8. However, this inhibitor did not affect chemical-induced damage to mitochondria. Therefore, whereas the apical caspase in receptor-mediated apoptosis seems to be caspase-8, chemical-induced apoptosis seems to act predominantly via a mitochondrial/caspase-9 pathway, with caspase-9 acting as the executioner rather than the initiator of apoptosis.

Resveratrol was proposed to induce apoptosis in the human myeloid leukemia line HL60 by up-regulating the expression of CD95L, which subsequently triggered CD95-mediated death (15). The question remained whether resveratrol might induce apoptosis in leukemia cells that are resistant to activation of the CD95 pathway. Many ALL cells, particularly of the B-lineage, are resistant to apoptosis mediated through CD95 (16). In this study, we used CD95-resistant pro-B and pre-B cell lines and CD95-sensitive T-lineage lines derived from patients with ALL to analyze the cytotoxicity of resveratrol and to evaluate the mechanism of cytotoxic action. We show that resveratrol can induce extensive apoptotic cell death in both CD95-sensitive and CD95-resistant ALL cells without significant toxicity to normal PBMCs. The mechanism of apoptosis induction by resveratrol was independent of the CD95-pathway in all leukemia lines tested and involved depolarization of mitochondrial membranes and activation of caspase-9. These data suggest that resveratrol may be useful as a novel, chemotherapeutic agent in the treatment of ALLs that are resistant to CD95-mediated apoptosis.

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³ The abbreviations used are: CD95L, CD95 ligand; DISC, death-inducing signal complex; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; ALL, acute lymphoblastic leukemia; PBMC, peripheral blood mononuclear cell; FACS, fluorescence-activated cell sorter; PI, propidium iodide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $\Delta\Psi_{\text{mit}}$, mitochondrial membrane potential; RT-PCR, reverse transcription-PCR; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide.

MATERIALS AND METHODS

Cell Culture. SEM, RS4;11, and MV4;11 lines were established from patients diagnosed with pro-B ALL containing the chromosomal translocation t(4;11)(q21;q23) (17–19). The presence of the t(4;11) translocation was confirmed in these cell lines by PCR, as described previously (20). The three pro-B ALL lines, the pre-B leukemia cells REH and Nalm-6, the T cell leukemia cells Jurkat and CEM, and HL60 myeloid leukemia cells were maintained at 37°C in 5% CO₂ in RPMI 1640 (Life Technologies, Inc., Karlsruhe, Germany) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, 1 mM sodium pyruvate, and 2 mM L-glutamine (Life Technologies, Inc.). PBMCs were isolated from healthy volunteers by Ficoll hypaque density centrifugation. PBMCs were cultured at a density of 0.5×10^6 cells/ml in RPMI 1640, as described above.

Flow Cytometric Analysis. Immunofluorescence analysis was performed on a FACSCalibur FACS using CELLQuest software (Becton Dickinson, Mountain View, CA). The following antibodies were used to determine the expression levels of CD95: phycoerythrin-conjugated antihuman CD95, clone DX2 (PharMingen, Hamburg, Germany), phycoerythrin-conjugated mouse IgG1 (Dako Diagnostika, Hamburg, Germany). Cell death was measured by lysing the cells in a hypotonic solution containing 1 mg/ml sodium citrate, 0.1% Triton X-100, and 50 µg/ml PI (Sigma Chemical Co., Deisenhofen, Germany) and analyzing the resulting nuclei by FACS as described previously (21). The extent of cell death (percentage) was determined by measuring the fraction of nuclei that contained sub-diploid DNA content. Fifteen thousand events were collected for each sample analyzed for sub-diploid nuclei.

To determine whether cell death was attributable to apoptosis, whole cells were stained with FITC-conjugated Annexin V (PharMingen) and PI (in PBS) according to the manufacturer's protocol. All analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregated cells. Fifteen thousand events were collected for each sample stained with Annexin V.

Sensitivity to CD95-mediated Death. For determination of sensitivity to CD95L-induced cell death, the soluble Fas Ligand kit (Alexis Corporation, Gruenberg, Germany) was used. Cells were plated in 96-well plates at a density of 0.5×10^6 cells/ml. A final concentration of 50 ng/ml soluble FLAG-tagged CD95L plus 1 µg/ml anti-FLAG antibody (enhancer) were added to the cells. Control cells were treated with enhancer only. Cells were incubated at 37°C for 20 h and then analyzed for the percentage of cell death by PI staining and FACS.

Resveratrol Treatment. Resveratrol was purchased from Sigma Chemical Co. and dissolved in methanol before use. Cells were split to a density of 0.5×10^6 /ml and treated with resveratrol at the indicated concentrations and for the indicated times. For time course experiments, the cells were treated once, and aliquots of each treatment group were taken each day for 4 days and analyzed by FACS for the percentage of cell death by PI staining.

For prolonged, multiple-dose treatment over a 9-day period, the cell density of leukemic cells and PBMCs was adjusted every 3 days to 0.5×10^6 /ml, and a final concentration of 50 µM resveratrol was added to the cells in fresh complete medium. Equivalent amounts of methanol were always added to cells in each experiment as a control for resveratrol-specific cell death (designated 0 µM or untreated cells). Aliquots of cells were taken every 3 days for analysis of cell death by PI staining.

Inhibition of CD95-CD95L Interactions. Cells were preincubated for 1 h at 37°C with nonspecific mouse IgG1 (Dako Diagnostika, Hamburg, Germany) or anti-CD95 antibody, clone ZB4 (Coulter Immunotech, Marseille, France) at a final concentration of 1 µg/ml. Control medium with methanol or 50 µM resveratrol was then added. After 24 h, SEM, MV4;11, Jurkat, Nalm-6, and HL60 cells were analyzed by FACS for the percentage of cell death. For RS4;11, REH, and CEM cells, fresh antibody solution was added at the above final concentration after 24 h, and the percentage of cell death was measured after 48 h. As a control for the efficient inhibition of CD95-CD95L interaction and apoptosis signaling, Jurkat T cells were preincubated with the above antibody solutions in separate wells for each experiment and then incubated with 50 ng/ml soluble CD95L plus 1 µg/ml enhancer for 24 h before FACS analysis.

RT-PCR. Total RNA was isolated from cells using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's recommendations. First-

strand cDNA synthesis was performed with either 1 µg or 5 µg total RNA. In separate experiments, total RNA was reverse-transcribed to cDNA using either 1 µg pd(N)₆ random hexamer primers (Amersham Pharmacia Biotech) or 0.5 µg oligo(dT) primer (Life Technologies, Inc.); 200 units Superscript II reverse transcriptase (Life Technologies, Inc.); 10 mM DTT (Life Technologies, Inc.); 0.5 mM each of dTTP, dGTP, dCTP, and dATP (Amersham Pharmacia Biotech); and 1× first-strand synthesis buffer (Life Technologies, Inc.) containing 50 mM Tris-Cl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂, final concentration. PCR reactions consisted of 3 µl cDNA, 0.2 µM each 5' and 3' primer; 1.5 mM MgCl₂; 0.2 mM each of dTTP, dGTP, dCTP, and dATP; 2.5 units TAQ polymerase (Life Technologies, Inc.); and 1× PCR reaction buffer (Life Technologies, Inc.). The following oligonucleotide primer sequences were used: CD95L, 5' primer (5'-AGCCAGATGCACACAGCATC), CD95L, 3' primer (5'-TACAACATTCTCGGTGCCTG); GAPDH, 5' primer (5'-CT-TACCACCATGGAGAAGG); and GAPDH, 3' primer (5'-CCTGCTTCAC-CACCTTCTTG). PCR amplification was performed using the following parameters: 94°C, 2 min; 34 cycles of 94°C, 30 s; 58°C, 45 s; and 72°C, 60 s, and then a final extension of 8 min at 72°C.

Analysis of Mitochondrial Membrane Potential. JC-1 dye was purchased from Molecular Probes (Leiden, The Netherlands) and stored in DMSO (Sigma Chemical Co.). A working stock solution was made in ethanol at a concentration of 0.5 mg/ml. Cells were adjusted to a density of 0.5×10^6 /ml and stained with 2 µg/ml JC-1 for 30 min at 37°C. Cells were then washed twice with cold PBS (Sigma Chemical Co.) containing 1 g/liter BSA (Carl Roth, Karlsruhe, Germany) and 7 mM sodium azide. Cells were resuspended in wash buffer and analyzed by FACS.

Activation of Caspase-9. Activation of caspase-9 was determined by colorimetric assay using the caspase-9 activation kit from R&D Systems (Wiesbaden-Nordenstadt, Germany) following the manufacturer's protocol. The cells were incubated for either 48 or 72 h with and without resveratrol. All protein lysates were precleared by centrifugation at $5200 \times g$ for 5 min at 4°C. Lysates were incubated with and without caspase-9 substrate for 5 h at 37°C before measurement on a microplate reader set at a wavelength of 405 nm. The relative increase in caspase-9 activity in resveratrol-treated cells compared with untreated cells was calculated after subtracting the background measurement obtained from lysates containing no substrate.

Inhibition of Caspase Activation. The broad spectrum caspase inhibitor Z-VAD-FMK (Calbiochem, Schwalbach, Germany) was dissolved in DMSO. Cells were pretreated with either medium containing DMSO or inhibitor for 2 h at the concentrations indicated. Control medium or medium containing resveratrol was then added to achieve a final concentration of 50 µM resveratrol. The cells were analyzed for changes in mitochondrial membrane potential using JC-1 dye and for cell death using PI staining of nuclei.

Statistical Analysis. All statistical analyses were performed with GraphPad software (GraphPad Software, Inc., San Diego, CA), and data were displayed as arithmetic means \pm SE. *P*s were obtained using two-tailed paired *t* tests with a confidence interval of 95% for evaluation of the significance of differences between treatment groups.

RESULTS

Resveratrol Induces Apoptosis in the t(4;11) Lines. Time course and dose-response analyses were performed on three pro-B t(4;11) ALL lines, SEM, RS4;11, and MV4;11; the pre-B leukemia lines REH and Nalm-6; the T-ALL lines CEM and Jurkat; and HL60 myeloid cells to determine the cytotoxicity of resveratrol for each line. The cells were treated once with 0, 1, 10, 25, 50, 75, and 100 µM resveratrol, and the fraction of nonviable cells (percentage of cell death) was assessed over a period of 4 days by PI staining of nuclei and evaluation by FACS (Fig. 1). The leukemia lines showed variability in sensitivity to resveratrol-induced death. MV4;11, Nalm-6, Jurkat, and HL60 cells displayed the greatest sensitivity to the cytotoxic action of resveratrol, with >60% cell death after 4 days. The two pro-B lines, SEM and RS4;11; the pre-B line, REH; and the T cell ALL line, CEM, were more resistant to the cytotoxic action of resveratrol and displayed <40% cell death after one initial treatment.

To determine whether the resveratrol-induced cell death observed

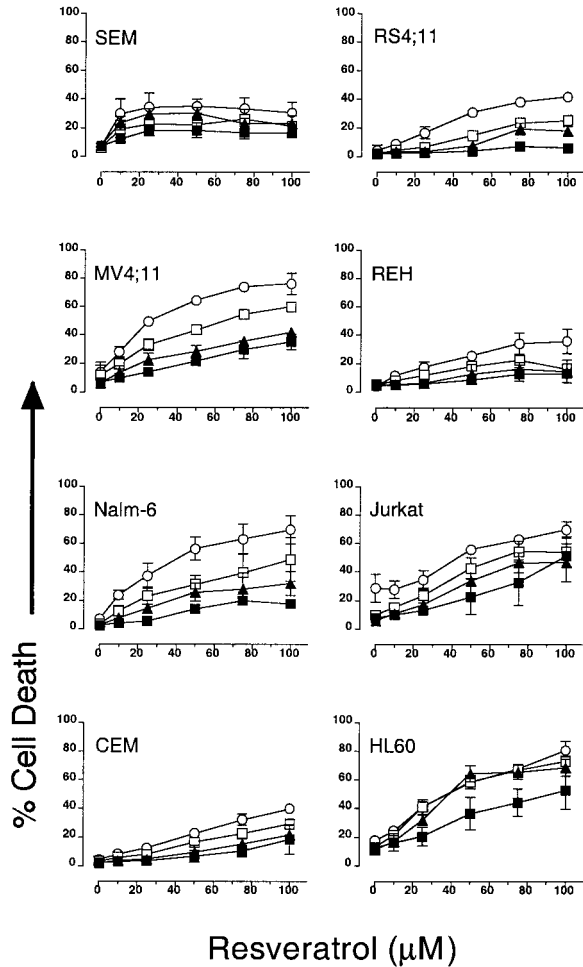


Fig. 1. Resveratrol is cytotoxic to the leukemia cell lines. The leukemia lines were treated with 0, 1, 10, 25, 50, 75, and 100 μM resveratrol. Aliquots of cells were analyzed each day for percentage of cell death by PI staining and FACS over a period of 4 days. ■, day 1; ▲, day 2; □, day 3; and ○, day 4. The data presented represent the mean of the percentage of cell death \pm SE from three separate experiments.

in the leukemia lines was attributable to apoptosis, the leukemic cells were treated with resveratrol and then stained with FITC-conjugated Annexin V plus PI. Exposure of the membrane phospholipid phosphatidylserine to the external cellular environment is one of the earliest markers of apoptotic death (22). Annexin V is a protein that binds to phosphatidylserine and can be used to detect early stages of apoptosis (23). PI, which does not enter cells with intact membranes, was used to differentiate between early apoptotic (Annexin V-positive) and late apoptotic or necrotic cells (Annexin V-PI-double positive). All eight leukemia lines showed a distinct population of early apoptotic cells that stained only with FITC-Annexin V but not with PI (Fig. 2, *bottom right quadrant*). Early apoptotic populations of cells were observed by 24 h in all lines except REH and RS4;11 cells, which required 48- and 72-h treatment times, respectively, for a clear early apoptotic population to be observed. Cells staining with both Annexin V and PI were present (Fig. 2, *top right quadrant*) and represented later stages of apoptosis and cells undergoing rapid secondary necrosis in culture. These results showed that resveratrol induced apoptotic cell death in each leukemic line.

Multiple Dose Treatment of the Leukemic Cells and Analysis of Cytotoxicity in Normal PBMCs. To determine the optimal dose that would be effective in killing the leukemic cells but would not substantially affect normal blood cells, PBMCs were isolated from healthy volunteers and treated with increasing doses of resveratrol as

described above. At concentrations of 75 and 100 μM resveratrol, a significant amount of cell death in normal PBMCs was observed after 4 days of treatment compared with the untreated cells ($P = 0.0354$ and 0.0002, respectively; Fig. 3A). However, at 50 μM or less resveratrol, there was not a statistically significant difference in the percentage of cell death between the untreated and treated PBMCs. Therefore, 50 μM resveratrol was used in all subsequent analyses of cell death induced by resveratrol.

The treatment time of the leukemic cells was extended and the

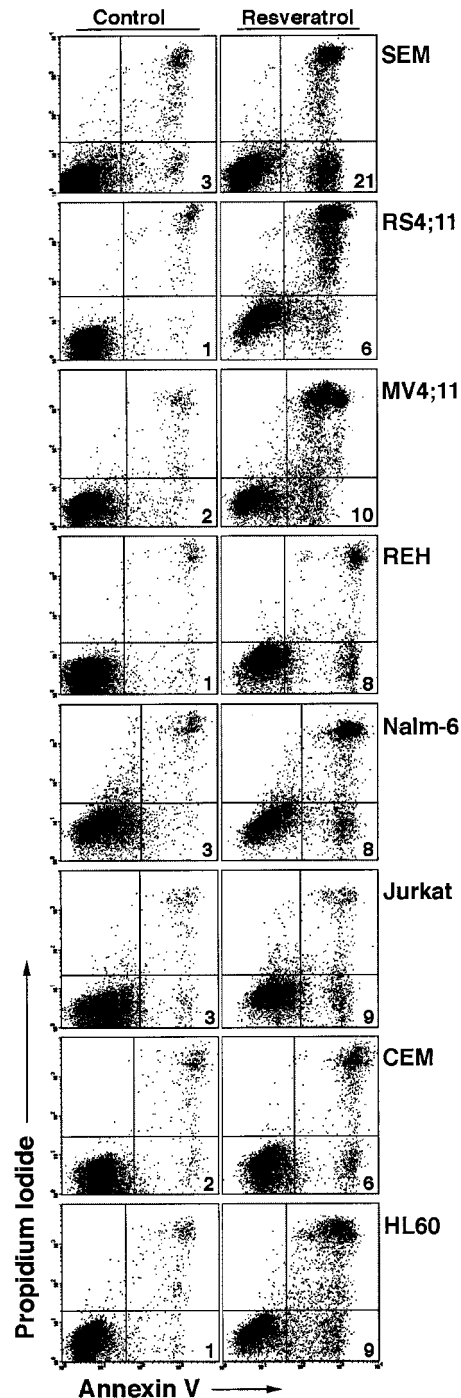


Fig. 2. Resveratrol induces apoptosis in acute leukemia cells. Whole cells were treated with 0 or 50 μM resveratrol and stained with Annexin V and PI. Numbers in the *bottom right quadrant* of each dot plot represent the percentage of cells in early apoptosis (Annexin V-positive and PI-negative) for untreated and resveratrol-treated cells. The data are representative of three separate experiments.

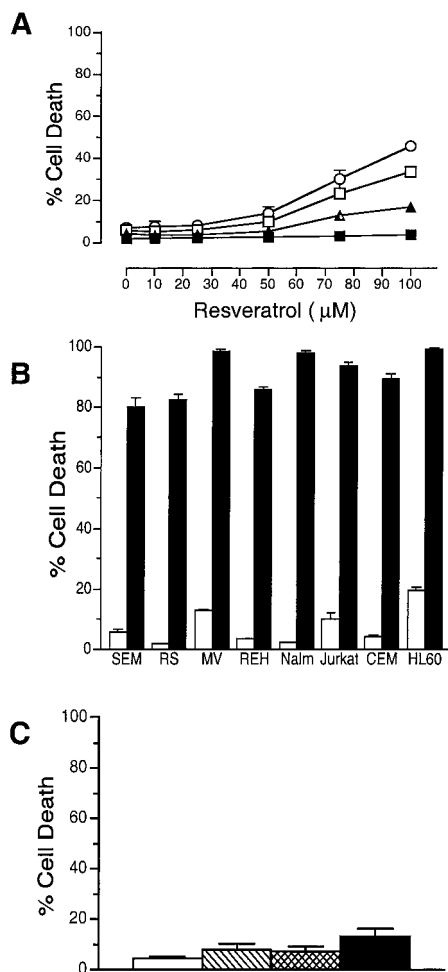


Fig. 3. Multiple doses of 50 μM resveratrol efficiently killed the leukemic cells with minimal cytotoxicity against normal PBMCs. *A*, normal PBMCs were treated with 0, 1, 10, 25, 50, 75, and 100 μM resveratrol. Aliquots of cells were analyzed as described in the legend to Fig. 2 over a period of 4 days. ■, day 1; ▲, day 2; □, day 3; and ○, day 4. The data presented represent the mean of the percentage of cell death \pm SE from three healthy volunteers. *B*, the leukemic cells were initially treated with 0 (□) and 50 μM (■) resveratrol, and fresh resveratrol at the same concentration was added every 3 days for a total of 9 days. The data represent the percentage of cell death \pm SE observed after 9 days of treatment from three separate experiments. *C*, normal PBMCs were treated with 0 and 50 μM resveratrol, and fresh resveratrol was added every 3 days for a total of 9 days. Aliquots of cells were analyzed for percentage of cell death by PI staining and FACS. The data represent the arithmetic mean \pm SE for untreated cells (□) and resveratrol-treated PBMCs on day 3 (▨), day 6 (▩), and day 9 (■) from three healthy donors.

cytotoxic effects of multiple doses of resveratrol on the leukemic cells and normal PBMCs were compared under identical conditions. All cells were retreated with 0 or 50 μM resveratrol every 3 days for a total of 9 days and analyzed for the percentage of cell death by FACS. After 9 days of multiple treatments, all leukemia lines exhibited 80–99% cell death, which was statistically significant compared with untreated cells ($P < 0.05$, Fig. 3*B*). Thus, resveratrol was clearly effective in killing the leukemic cells. Analysis of PBMC mortality after the extended 9-day treatment period with 50 μM resveratrol revealed $\sim 13.5 \pm 2.8\%$ cell death compared with $5.5 \pm 2.1\%$ (\pm SE) for the untreated PBMCs (Fig. 3*C*), and this difference was not statistically significant ($P = 0.169$).

Resveratrol-induced Apoptosis Is Not Mediated by CD95-CD95L Interactions. Several reports have appeared in the last few years describing the anticancer activity of resveratrol (2, 24, 25). Clement *et al.* (15) have suggested that resveratrol acts by inducing the expression of CD95L and triggering CD95-signaling-dependent apoptosis in HL60 myeloid leukemia cells. However, many acute

leukemias are resistant to CD95-mediated apoptosis, especially ALL of the B-cell lineage (16). Fig. 4 shows the sensitivity of each line to CD95-mediated cell death. Although all lines expressed CD95 on the cell surface (data not shown), the three pro-B t(4;11) ALL lines, SEM, RS4;11, and MV4;11, the pre-B leukemia lines REH and Nalm-6, and HL60 myeloid cells were completely resistant to CD95-mediated death when incubated with CD95L (Fig. 4). Only the two T-cell leukemia lines, Jurkat and CEM, showed a statistically significant difference in the percentage of cell death ($P < 0.05$) between the control cells and CD95L-treated cells, and these lines were used as positive controls for CD95-CD95L interaction in this experiment. No correlation between resistance to CD95-signaling and the cytotoxic actions of resveratrol was observed (Figs. 1 and 4). MV4;11, Nalm-6, and HL60 cells were resistant to CD95-mediated death, but displayed 2-fold or greater sensitivity to resveratrol than CEM cells, which were sensitive to CD95L-induced death. Furthermore, Jurkat cells were sensitive to both CD95L-induced death and the cytotoxic action of resveratrol.

Clement *et al.* (15) suggested resveratrol could increase CD95L mRNA expression. After treating HL60 myeloid cells with 0 and 32 μM resveratrol, these authors performed RT-PCR to analyze the expression of CD95L. To determine whether resveratrol could affect levels of CD95L in the eight leukemia lines, we analyzed mRNA levels of CD95L by RT-PCR in untreated cells and cells treated with 50 μM resveratrol. RNA from Jurkat cells treated with phorbol 12-myristate 13-acetate plus ionomycin were used as a positive control for CD95L in the RT-PCR. Fig. 5 shows the results of the RT-PCR analysis. The levels of CD95L transcript were exceedingly low in the HL60 cells and the ALL lines compared with the Jurkat control cells. We did not observe a change in levels of CD95L transcript between untreated and resveratrol-treated SEM, RS4;11, REH, Nalm-6, and HL60 cells. Only MV4;11, Jurkat, and CEM showed a small increase in CD95L PCR signal after treatment with resveratrol. In an attempt to reproduce the results from Clement *et al.* (15), HL60 cells also were treated with control medium and 32 μM resveratrol for 20 h and analyzed by RT-PCR. No difference in levels of CD95L mRNA was observed between untreated and HL60 treated with either 32 μM or 50 μM resveratrol.

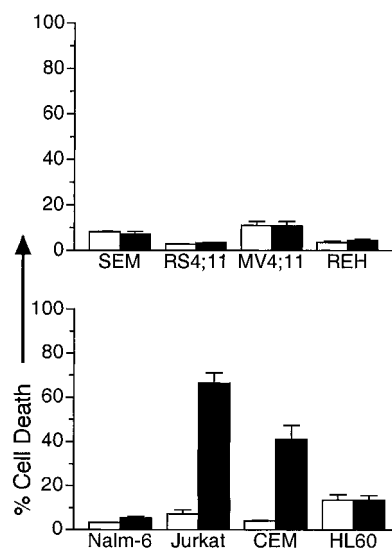


Fig. 4. B-lineage and myeloid leukemia lines are resistant to CD95L-mediated cell death. Leukemic cells were treated with 1 $\mu\text{g}/\text{ml}$ enhancer only as a control (□) or enhancer + 50 ng/ml soluble CD95L (■) for 20 h. The cells were lysed in a hypotonic solution containing PI and analyzed by FACS. The percentage of cell death was determined by measuring the fraction of nuclei that contained sub-diploid DNA content. The data presented represent the arithmetic mean of the percentage of cell death \pm SE from three separate experiments.

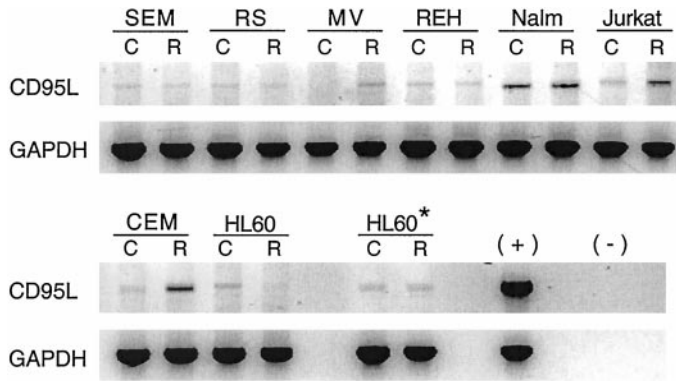


Fig. 5. RT-PCR of CD95L in untreated and resveratrol-treated leukemia cells. RNA was isolated from leukemia cells treated with control medium (C) or 50 μ M resveratrol (R) for 24 h, and 1 μ g of RNA was reverse transcribed into cDNA using random hexamer primers. Three μ l of each cDNA reaction were then used in the PCR reactions. Similar results were obtained using 5 μ g of RNA and oligo(dT) primers to generate cDNA. HL60 marked with *, RT-PCR reactions generated from 5 μ g of RNA isolated from cells treated with 0 and 32 μ M resveratrol for 20 h, as described previously (15). Jurkat T cells treated with phorbol 12-myristate 13-acetate and ionomycin were used as a positive control (+) for CD95L, water was used in the place of cDNA as a negative control (-), and as a control for equal reaction efficiency and gel loading, oligonucleotides specific for the housekeeping gene *GAPDH* were used. CD95L and *GAPDH* primers produced 553-bp and 491-bp PCR products, respectively.

To examine further whether the mechanism of resveratrol action involved triggering the CD95-CD95L pathway in these leukemic cells, the leukemic cells were preincubated for 1 h with a nonspecific IgG1 antibody or the antagonistic anti-CD95 antibody, clone ZB4, known to block signaling by CD95L. Initially, the cells were preincubated for 1 h with 0.5 μ g/ml antibody and then treated with control medium or 50 μ M resveratrol. In these initial experiments, we observed no difference in the percentage of cell death induced by resveratrol in the presence or absence of the ZB4 blocking antibody. To determine whether a higher dose of antibody and/or a lower concentration of resveratrol was necessary to observe blocking of CD95-CD95L interactions, we then used 1 μ g/ml ZB4 antibody in the presence or absence of 25 μ M and 50 μ M resveratrol. SEM, MV4;11, Jurkat, Nalm-6, and HL60 were analyzed by FACS for the percentage of cell death after 24 h (Fig. 6A). RS4;11, REH, and CEM cells, which were initially more resistant to the cytotoxic action of resveratrol than the other lines, were incubated further with fresh antibody solutions after 24 h and analyzed for cell death after a total of 48 h (Fig. 6A). As a positive control to ensure that the anti-CD95 antibody was effectively blocking CD95-CD95L interactions in each experiment, Jurkat T cells were preincubated with antibody for 1 h in separate wells and then treated with soluble CD95L. The Jurkat cells were analyzed for CD95L-mediated death after 24 h, and these data showed that the antibody was effectively blocking signaling through CD95 in each experiment (Fig. 6B). In the B-lineage ALL lines, CEM, and HL60 cells, no difference in resveratrol-induced cell death was observed when CD95-signaling was blocked compared with cells preincubated with control IgG1 (Fig. 6A). Furthermore, resveratrol did not trigger an increase in cell surface expression of CD95 or increase the sensitivity of the leukemic lines to CD95-mediated apoptosis (data not shown). Interestingly, Jurkat T cells treated with 50 μ M resveratrol showed an increase in cell death from $12.7 \pm 2.7\%$ to $32.0 \pm 6.6\%$ (\pm SE, $P = 0.0184$) when CD95-signaling was blocked by 1 μ g/ml antagonistic antibody (Fig. 6A). Taken together, we conclude that CD95-CD95L interactions and signaling are not required for the induction of apoptosis by resveratrol in these leukemia lines.

Resveratrol Disrupts Normal Mitochondrial Membrane Potential. Recently, distinct caspase cascades were characterized for receptor *versus* chemical-induced apoptosis, indicating chemical-induced

apoptosis is mediated by the mitochondria/caspase-9 activation pathway (14). To delineate the mechanism of resveratrol cytotoxicity in the acute leukemia lines, the lipophilic cation JC-1 was used to determine whether resveratrol induced alterations in mitochondrial membrane potential in these cells. JC-1 has been used successfully for flow cytometric measurements of mitochondrial potential because of its dual emission characteristics that are sensitive to membrane potential (26). JC-1 is mitochondria-selective and forms aggregates in normal polarized mitochondria that result in a green-orange emission of 590 nm after excitation at 490 nm. However, the monomeric form present in cells with depolarized mitochondrial membranes emits only green fluorescence at 527 nm. The leukemia cells were untreated or treated with resveratrol for 24 h, stained with JC-1, and analyzed by FACS. Fig. 7 shows a clear increase in the percentage of cells (*lower right quadrants*) that emitted only green fluorescence after resveratrol

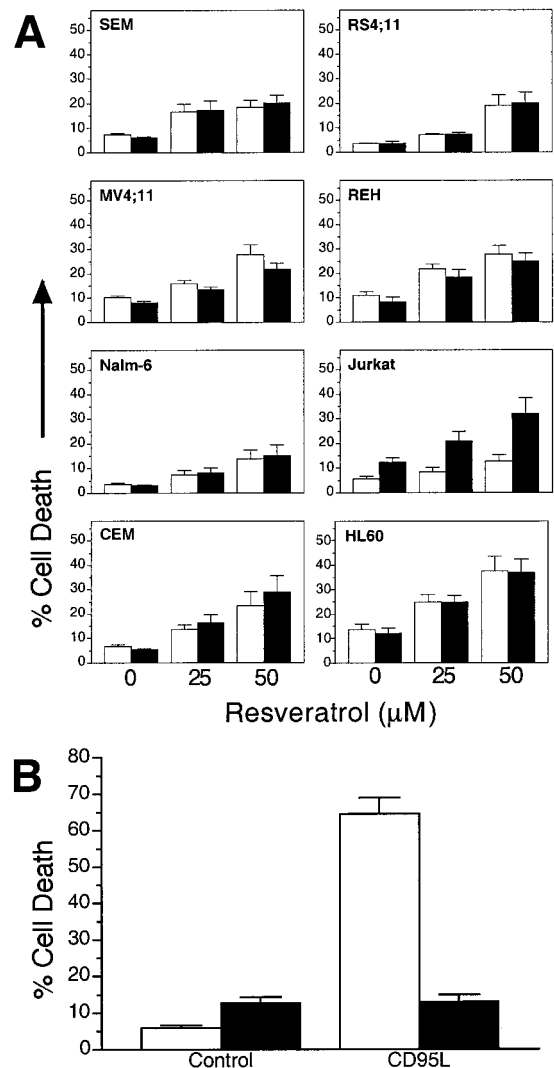


Fig. 6. CD95-CD95L interactions are not required for resveratrol-mediated death. A, the leukemic cells were preincubated for 1 h with nonspecific IgG1 (\square) or anti-CD95 clone ZB4 (\blacksquare) at a concentration of 1.0 μ g/ml. Then resveratrol was added, resulting in final concentrations of 0, 25, and 50 μ M. SEM, MV4;11, Nalm-6, Jurkat, and HL60 cells were incubated for 24 h before analysis of cell death by PI staining and FACS. RS4;11, REH, and CEM were analyzed after 48 h incubation with blocking antibody. Data presented represent the arithmetic mean \pm SE from four separate experiments. B, for each experiment with resveratrol, Jurkat T cells were preincubated with IgG1 (\square) or clone ZB4 (\blacksquare) in separate wells as a control for blocking of CD95-CD95L interactions, as described above. After 1 h, PBS or soluble CD95L + enhancer were added to the cells, and the percentage of cell death was measured by PI staining and FACS after 24 h. Data presented represent the arithmetic mean \pm SE from five separate experiments.

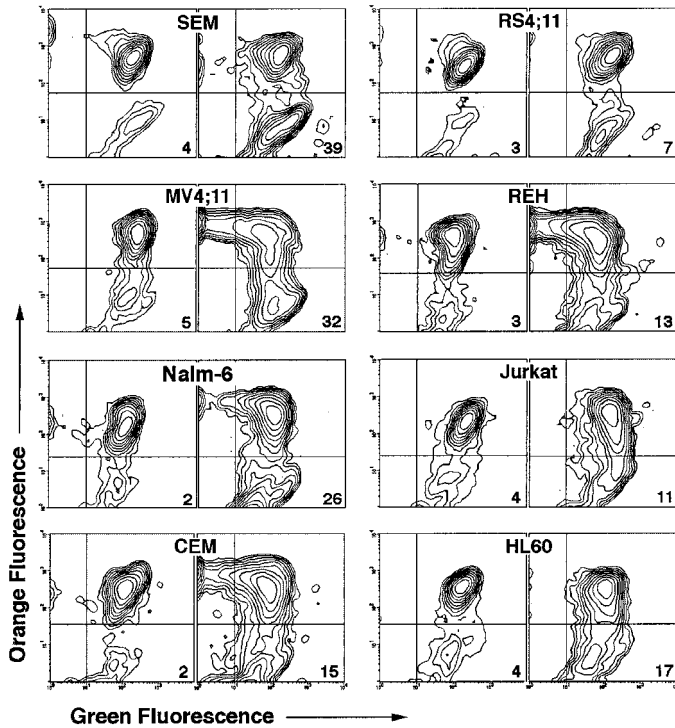


Fig. 7. Resveratrol induces depolarization of mitochondrial membranes. Each cell line was treated with 0 (left plot) and 50 μM (right plot) resveratrol for 24 h and stained with the mitochondria-selective JC-1 dye. Cells with normal polarized mitochondrial membranes emit green-orange fluorescence (top right quadrant). The number in the bottom right quadrant of each dot plot represents the percentage of cells that emit only green fluorescence attributable to depolarized mitochondrial membranes.

treatment, representing cells with depolarized mitochondrial membranes. Also, in MV4;11, REH, and CEM cells treated with resveratrol, a population of cells with hyperpolarized mitochondrial membranes was present, as indicated by the cells emitting only red fluorescence (upper left quadrant). However, the significance of this hyperpolarization response induced by resveratrol is not clear. The number of cells with loss of or reduced mitochondria membrane potential ($\Delta\Psi_{\text{mt}}$) increased over time after the addition of resveratrol (Table 1) and was dose-dependent for all leukemia lines (data not shown). Table 1 compares the kinetics of resveratrol-induced disruption of $\Delta\Psi_{\text{mt}}$ with cell death over 3 days. These data suggest that disruption of mitochondrial membranes by resveratrol coincides with the presence of apoptotic death and, in some cases, appears to precede cell death.

Resveratrol Activates Caspase-9. Caspase-9 was reported to be the apical caspase in the initiation of the caspase cascade during

chemical-induced apoptosis (14). Therefore, the activation status of caspase-9 was analyzed after the addition of resveratrol to the leukemia lines. Low levels of caspase-9 activity were already observed in some lines 24 h after the addition of resveratrol (data not shown). However, maximum caspase-9 activation was observed well after the beginning of resveratrol-induced changes in mitochondrial membrane potential. Caspase-9 activation increased ~ 4 – 9 -fold after 48 h treatment of SEM, MV4;11, Nalm-6, Jurkat, and HL60 cells and 72 h treatment of RS4;11, REH, and CEM cells with resveratrol (Fig. 8).

Additional evidence that resveratrol acts via a mitochondrial/caspase-9 pathway rather than by upstream activation of caspase-8 was obtained using the broad spectrum caspase inhibitor Z-VAD-FMK. We found that relatively high concentrations of inhibitor (100–200 μM) were necessary to block resveratrol-mediated death in the leukemia lines over the 2–3-day treatment periods. Cells were pre-treated with inhibitor for 2 h before the addition of resveratrol, and changes in mitochondrial membrane potential and cell death were analyzed by JC1 and PI staining, respectively. As a control for inhibition of caspase-8 activation, Jurkat T cells were preincubated with inhibitor for 2 h and then treated with soluble CD95L. Fig. 9 shows the percentage of cells with depolarized mitochondrial membranes induced by resveratrol in the presence or absence of the Z-VAD-FMK inhibitor. No statistically significant changes were observed in the depolarizing activity of resveratrol for SEM, MV4;11, Nalm-6, Jurkat, CEM, and HL60 cells in the presence or absence of the broad spectrum caspase inhibitor, indicating resveratrol acts independently of upstream caspase activation. Interestingly, the two B-lineage cell lines RS4;11 and REH showed a substantial increase in

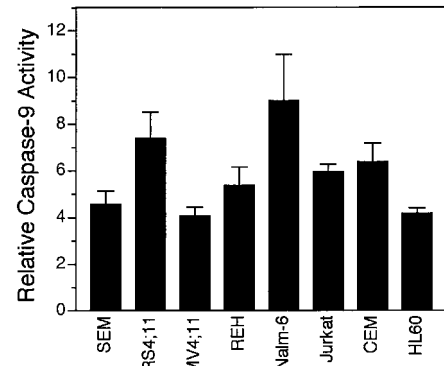


Fig. 8. Caspase-9 is activated by resveratrol. Cells were treated with 0 and 50 μM resveratrol, and protein lysates were prepared after 48 or 72 h. Lysates were incubated with and without caspase-9 substrate for 5 h at 37°C and measured at 405 nm on a microplate reader. The relative activity was determined by calculating the fold increase of caspase-9 activity in resveratrol-treated cells over the activity in untreated cells. Data represent the arithmetic mean \pm SE of three separate experiments.

Table 1 Progressive loss of mitochondrial membrane potential ($\Delta\Psi_{\text{mt}}$) relative to percentage of cell death induced by resveratrol

The cells were cultured in the presence of 50 μM resveratrol. At the indicated time points, the percentage of cells with depolarized mitochondrial membranes was determined by JC-1 staining and subsequent FACS analysis. The percentage of cell death was determined by PI staining of the nuclei and analysis of the sub-diploid population by FACS. All values represent the arithmetic mean \pm SE from three independent experiments.

Cell line	Day 1		Day 2		Day 3	
	% cells with red. $\Delta\Psi_{\text{mt}}$ ^a	% cell death	% cells with red. $\Delta\Psi_{\text{mt}}$	% cell death	% cells with red. $\Delta\Psi_{\text{mt}}$	% cell death
SEM	38.2 \pm 6.9	24.1 \pm 2.6	75.1 \pm 5.6	45.3 \pm 3.1	85.8 \pm 2.6	56.6 \pm 3.9
RS4;11	7.1 \pm 2.0	6.8 \pm 1.6	14.2 \pm 2.3	10.8 \pm 1.7	35.5 \pm 5.8	25.9 \pm 1.8
MV4;11	29.6 \pm 5.9	27.8 \pm 4.6	71.9 \pm 1.6	43.9 \pm 2.1	91.4 \pm 1.3	63.8 \pm 1.5
REH	5.8 \pm 1.1	4.1 \pm 0.6	12.5 \pm 2.5	9.5 \pm 1.3	20.7 \pm 3.3	24.7 \pm 2.6
Nalm-6	13.9 \pm 4.3	11.6 \pm 3.4	52.4 \pm 5.4	30.8 \pm 3.5	83.6 \pm 4.9	50.6 \pm 3.7
Jurkat	18.1 \pm 7.6	12.4 \pm 2.8	41.6 \pm 6.0	39.4 \pm 2.2	49.5 \pm 1.5	52.0 \pm 1.5
CEM	12.0 \pm 1.2	10.9 \pm 2.4	26.7 \pm 3.1	22.0 \pm 0.3	50.7 \pm 1.2	34.1 \pm 1.8
HL60	25.1 \pm 7.3	30.4 \pm 5.3	59.9 \pm 6.8	76.2 \pm 1.4	85.9 \pm 1.9	81.5 \pm 5.3

^a red. $\Delta\Psi_{\text{mt}}$, loss of or reduced mitochondrial membrane potential.

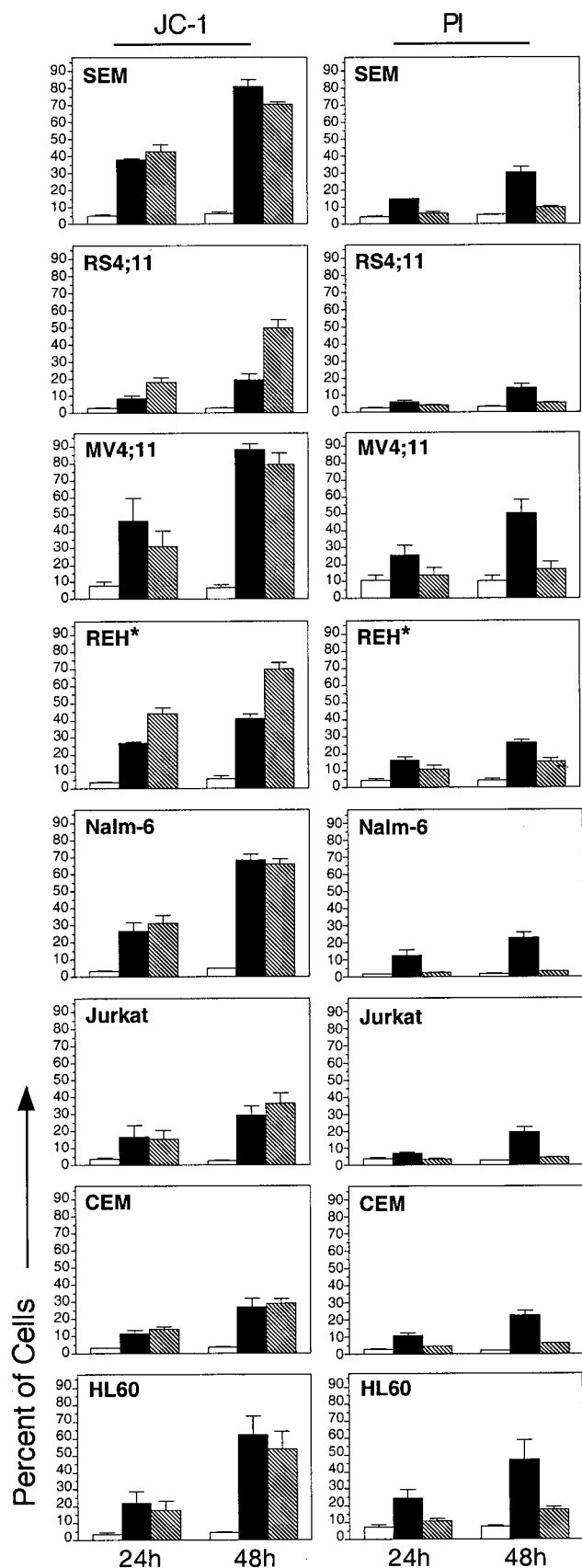


Fig. 9. The broad spectrum caspase inhibitor Z-VAD-FMK inhibits cell death but not depolarization of mitochondrial membranes induced by resveratrol. All leukemia lines were incubated with $100 \mu\text{M}$ Z-VAD-FMK inhibitor except MV4;11, REH, and HL60 cells, which were incubated with $200 \mu\text{M}$ inhibitor. Cells were preincubated with medium containing DMSO or inhibitor for 2 h and then treated with control medium or resveratrol for 24 h and 48 h. REH cells were analyzed after 48 h and

cells with depolarized mitochondrial membranes in the presence of resveratrol and Z-VAD-FMK, further indicating that resveratrol is capable of acting directly on the mitochondria rather than through upstream activation of caspases. Analysis of resveratrol-induced cell death revealed Z-VAD-FMK completely blocked death in all lines except REH, where only a partial block was observed. Jurkat cells treated with this inhibitor and soluble CD95L showed caspase-8 activation induced by CD95-CD95L interaction was effectively blocked in these experiments (Fig. 10). The Z-VAD-FMK inhibitor completely blocked both depolarization of mitochondria and cell death induced upstream by caspase-8 activation in the Jurkat control cells. From these data, we conclude that resveratrol induces apoptosis in the ALL and myeloid lines by a mechanism that involves the depolarization of mitochondrial membranes and the activation of caspase-9.

DISCUSSION

It was suggested by Clement *et al.* (15) that resveratrol triggered CD95-dependent apoptosis by up-regulating expression of CD95L in HL60 myeloid cells. These authors reported that resveratrol increased the expression of CD95L in HL60 cells, and resveratrol-mediated cell death in these cells was inhibitable by treatment with both anti-CD95L and anti-CD95 (clone ZB4) antibodies. A role for CD95-CD95L interactions in chemotherapy-induced apoptosis has been proposed and challenged over the last several years. It was recently proposed that CD95L-induced DISC formation and activation of the apoptotic program by chemotherapeutic agents (in this case, doxorubicin) may be important in the early phase of drug-induced apoptosis, and that later stages of apoptosis may be attributable to other effects (13). In contrast to the data presented by Clement *et al.*, we were unable to observe an increase in CD95L in HL60 cells in response to resveratrol treatment by RT-PCR. Our analysis of mRNA transcripts by RT-PCR (Fig. 5) revealed no change in CD95L in HL60 cells after treatment with resveratrol. In our experiments, the use of the antagonistic antibody ZB4 did not show inhibition of resveratrol-induced cell death in the eight leukemia lines, including HL60 cells, although we could clearly show that this antibody blocked CD95L-mediated death in Jurkat (Fig. 6). The reasons for the discrepancy between our data and that of Clement *et al.* (15) remain unclear. These authors also showed that a breast carcinoma cell line displayed a substantial increase in viability when preincubated with anti-CD95 antibody compared with the isotype control in this study. Although we did not observe involvement of CD95-CD95L interactions in our study of eight leukemia lines treated with resveratrol, in certain cell types, such as breast carcinoma cells, it may be possible that resveratrol can act through CD95-mediated mechanisms.

Our investigation was focused mainly on B-lineage ALL, because they have been shown to be generally resistant to CD95-mediated apoptosis (16). In particular, the pro-B ALL lines SEM, RS4;11, and MV4;11 represent a subgroup of high-risk ALLs with translocation t(4;11) (27) that were previously shown by our lab to be refractive to CD95-signaling (20). From our data, we conclude that CD95-CD95L interactions do not initiate resveratrol-induced cell death in HL60 cells or in the ALL lines. These conclusions are now supported by several pieces of data: (a) resveratrol induces apoptosis in both CD95-sensitive and CD95-resistant leukemia cell lines, and no cor-

72 h (*). Fresh inhibitor was added to the cells every 24 h. □, control cells; ■, cells treated with $50 \mu\text{M}$ resveratrol alone; ▨, cells treated with inhibitor + resveratrol. Cells were analyzed for percentage of cells with depolarized mitochondrial membranes (left graphs) and percentage of cell death (right graphs) by JC-1 and PI staining, respectively. Data represent the arithmetic mean \pm SE of three separate experiments.

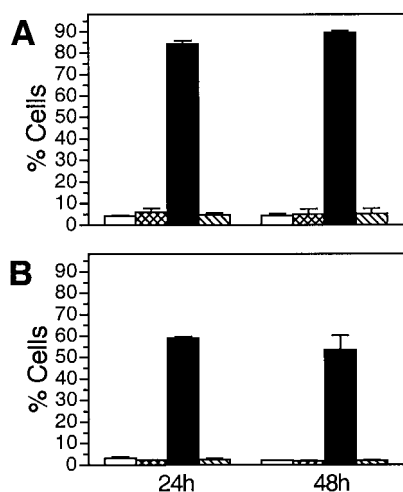


Fig. 10. Z-VAD-FMK inhibits both caspase-8-mediated depolarization of mitochondrial membranes and cell death. Jurkat cells were preincubated with medium containing DMSO or inhibitor at a final concentration of 100 μM , then either medium or soluble CD95L was added. Fresh inhibitor was added to the cells every 24 h. Cells were analyzed for percentage of cells with depolarized mitochondrial membranes and percentage of cell death after 24 h and 48 h. \square , control with DMSO; \blacksquare , control with inhibitor; \blacksquare , soluble CD95L and DMSO; \blacksquare , soluble CD95L with Z-VAD-FMK. A, percentage of cells with depolarized mitochondrial membrane. B, percentage of cells with sub-diploid nuclei (percentage of cell death). Data represent the arithmetic mean \pm SE of three separate experiments.

relation between resistance to CD95-signaling in the leukemia cells and the cytotoxic action of resveratrol was observed (Figs. 1 and 4); (b) resveratrol did not sensitize the CD95-resistant cell lines to CD95-signaling; (c) no change in the expression of CD95L was observed by RT-PCR in five of the cell lines, including HL60 cells (Fig. 5). Although MV4;11, CEM, and Jurkat cells showed what appeared to be a small increase in the steady-state level of CD95L mRNA, our RT-PCR analyses as well as those of Clement *et al.* (15) were not quantitative, and these data cannot be used to conclude that actual changes in transcription of CD95L mRNA have occurred; (d) an antagonistic antibody against CD95 failed to decrease death initiated by resveratrol in all eight lines (Fig. 6); and (e) we have used the broad spectrum caspase inhibitor Z-VAD-FMK to determine whether resveratrol-induced caspase activation occurred upstream or downstream of the depolarization of mitochondria (14). In these experiments, we could clearly show that depolarization of mitochondrial membranes induced by caspase-8 activation in CD95L-treated Jurkat cells was indeed blocked by Z-VAD-FMK (Fig. 10). This inhibitor could also block caspase activation leading to cell death after treatment of the cells with resveratrol. However, Z-VAD-FMK failed to block depolarization of mitochondrial membranes induced by resveratrol treatment in the eight lines, indicating that resveratrol-induced caspase activation occurred downstream of changes in the mitochondrial membranes, and depolarization was not dependent upon caspase-8 activity (Fig. 9). Two recent publications lend support for our conclusions that resveratrol acts through an apoptotic pathway which does not rely on CD95-CD95L interactions at early stages of cell death. Tsan *et al.* (28) showed resveratrol induced CD95-independent apoptosis in THP-1 monocytic leukemia cells. Using CEM cells and a caspase-8 deficient Jurkat cell line, Bernhard *et al.* (29) also showed that resveratrol-induced cell death in these cells was independent of CD95-mediated apoptosis.

In our analysis of the mechanistic action of resveratrol in the leukemia lines, we observed that this agent induced early changes in mitochondrial membrane potential that led to progressive depolarization culminating in apoptosis. At least three general mechanisms are known for mitochondria-mediated cell death: (a) disruption of elec-

tron transport and energy metabolism; (b) release of caspase-activating proteins; and (c) alteration of cellular reduction-oxidation potential (30). Resveratrol has been extensively studied for its antioxidant activities and was shown to directly destroy the tyrosyl radical on the R2 subunit of ribonucleotide reductase, an enzyme necessary for cellular proliferation (1, 4). Although the exact mechanism has not yet been defined, resveratrol was also recently shown to inhibit the F1 complex of the F0/F1-ATPase proton pump of the mitochondrial inner membrane, responsible for the synthesis of ATP from ADP in the oxidative phosphorylation pathway (31). The ability of resveratrol to interfere with the machinery of mitochondrial electron transport could be partially responsible for the depolarization of the mitochondrial membranes and the subsequent apoptosis observed in the resveratrol-treated ALL cells in our study.

In each ALL lines examined in our study, resveratrol induced the activation of caspase-9. Sun *et al.* (14) have recently characterized the order of caspase activation in receptor-mediated and chemical-induced apoptosis. Receptor-mediated apoptosis was dependent upon upstream activation of caspase-8, which was capable of activating the downstream caspases resulting in apoptosis. In etoposide-treated cells, however, caspase-9 acted as the apical caspase in the apoptotic cascade. Activation of caspase-9 occurs after the release of apoptogenic proteins, such as cytochrome *c*, from the mitochondria (30). Release of cytochrome *c* can occur with or without depolarization of the mitochondrial membranes by mechanisms that remain unclear. Cytochrome *c* forms a complex with Apaf-1 and caspase-9 in the cytosol, resulting in the activation of caspase-9 and the subsequent caspase-9-mediated activation of downstream caspases leading to apoptotic cell death (32, 33). Our data indicate that resveratrol acts through a mechanism involving the depolarization of mitochondrial membranes leading to the primary activation of caspase-9, which then initiates the caspase cascade resulting in apoptosis of the ALL cells. We clearly show that CD95-CD95L interactions were not important for initiating resveratrol-induced death, thereby excluding an upstream role for caspase-8 activation as the apoptosis-initiating event via receptor ligation. However, caspase-8 may be activated downstream in the caspase cascade after resveratrol treatment and may act to promote or amplify the apoptotic pathway at later stages (33).

In the present study, several cell lines derived from patients with ALL were used to determine whether resveratrol, a natural plant-derived product, was cytotoxic to these leukemic cells. Our studies showed that resveratrol can induce extensive apoptosis in the B-lineage and T-lineage ALL cells without significant cytotoxicity against normal PBMCs. Resveratrol was shown by others to have little toxicity against normal peripheral lymphocytes at a concentration of up to 32 μM after a 3-day treatment, although higher concentrations of the drug and longer treatment times were not tested (15). We found that concentrations of resveratrol above 50 μM were toxic to normal PBMCs, but 50 μM or less did not cause significant cell death in the normal blood cells over a period of 4 days. Furthermore, repeated administration of 50 μM resveratrol for up to 9 days was highly effective in inducing substantial apoptosis in all of the leukemic lines, again, with no statistically significant cytotoxicity observed against normal PBMCs. The results presented in this study suggest that resveratrol may be an effective drug with strong potential as a novel chemotherapeutic agent against ALL and may be particularly useful against B-lineage ALLs, including the t(4;11) subgroup, that are generally resistant to CD95-mediated death.

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Resveratrol Induces Extensive Apoptosis by Depolarizing Mitochondrial Membranes and Activating Caspase-9 in Acute Lymphoblastic Leukemia Cells

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