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Resveratrol inhibits the growth and induces the apoptosis of both normal and leukemic hematopoietic cells

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It is often postulated that *trans*-3,4',5-trihydroxystilbene (resveratrol, RES) exhibits cell growth regulatory and chemopreventive activities. However, mechanisms by which this polyphenol inhibits tumor cell growth, and its therapeutic potential are poorly understood. Using various human leukemia cells, we have first defined the anti-tumoral doses of this compound. RES inhibited the proliferation and induced the apoptosis of all tested lymphoid and myeloid leukemia cells with IC_{50} = 5–43 μM. Prior to apoptosis, RES-induced caspase activity in a dose-dependent manner and cell cycle arrest in G_2/M-phase, correlating with a significant accumulation of cyclins A and B. Leukemia cell death with RES required both caspase-dependent and -independent proteases, as it was significantly inhibited by simultaneous addition of Z-VAD-FMK and leupeptin to these cultures. While RES did not affect non-activated normal lymphocytes, this agent decreased the growth and induced the apoptosis of cycling normal human peripheral blood lymphocytes at lower concentrations (IC_{50} <8 μM) than those required for most leukemia cells. RES also induced the apoptosis of early normal human $CD34^+$ cells and decreased the number of colonies generated by these precursor cells in a dose-dependent manner (IC_{50} = 60 μM). Together, the data point to the complexity of RES-mediated signaling pathways and revealed the high anti-proliferative and proapoptotic activities of RES in normal cycling hemopoietic cells.

Introduction

In humans, the main polyphenol dietary sources are fruits and beverages (fruit juice, wine, tea, coffee, chocolate and beer) and, to a lesser extent vegetables, legumes and cereals (1,2). Polyphenols of the stilbenoid family are produced in plants in response to environmental stress such as adverse weather or insect, animal or pathogenic attack (3,4). Phenolic acids account for about one-third of the total intake and flavonoids (2) for the remaining two-thirds. The most abundant flavonoids in diet are flavanols (catechins and proanthocyanidins), anthocyanins and their oxidation products (2). Recently, various purified polyphenolic compounds were shown to display beneficial effects *in vitro* or *in vivo*, including anti-oxidant, chemopreventive and antitumoral properties (5–9). One of the polyphenolic phytoalexins, resveratrol (RES), was shown (9,10) to decrease the development of carcinogen-induced pre-neoplasic lesions in mouse mammary glands and to prevent tumor formation in mice. RES also represses different classes of androgens while increasing other factors including prostate-specific antigen, human glandular kallikrein-2 and the cyclin-dependent kinase inhibitor p21 (11,12). Anti-inflammatory effects of RES correlated with decreasing activity of cyclo-oxygenase, hydroperoxidase, NF-κB, AP-1 and their associated kinases (13–15).

Later observations asked RES therapeutic interest in human tumors or inflammatory diseases, and led us to investigate the hematotoxicity of this compound. RES has been reported to inhibit cell growth and to induce apoptosis in various human myeloid or lymphoid leukemia cell lines (15–19), but the precise mechanisms of anti-leukemic effect of RES and its toxicity on normal counterpart cells remain to be established. Recently, Gautam *et al.* (18) have suggested that leukemia cells were more sensitive than normal hematopoietic cells to the anti-proliferative effects of RES, a phenomenon that may help *ex vivo* leukemia cell purge from precursor cell preparations, prior to auto-transplantation. The present study aimed to define the anti-proliferative doses and the mechanism of action of RES in leukemia cells and the sensitivity of their normal hematopoietic counterpart to this compound.

Materials and methods

Chemicals

RES (*trans*-3,4',5-trihydroxystilbene) was purchased from Sigma-Aldrich, Saint Quentin Fallavier, France, or purified from Bordeaux red wine in the GESNIT laboratory (Université Victor Segalen Bordeaux 2, Bordeaux, France) as detailed elsewhere (20). RES is diluted in ethanol at 100 mM and used immediately to prevent *in vitro* alteration. Daunorubicin (RPR-Bellon, Neuilly sur Seine, France), leupeptin (Sigma-Aldrich) and Z-VAD-FMK (21) (Calbiochem, La Jolla, CA) were also used in this study.

Normal and leukemic cells

Human leukemia cell lines used in the present study were: myeloid K562, KCL22 (chronic myeloid leukemia-derived, Bcr-Abl^+), HL60 (promyelocytic) THP1, U937 (promonocytic), Jurkat (T lymphoid) and WSU-CLL (chronic lymphocytic leukemia-derived) B cells (22). Leukemia cell lines were maintained in RPMI-1640 medium supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml, 1 mM L-glutamine (Gibco BRL, Cergy-Pontoise, France) and 10% fetal calf serum (FCS; Eurobio, Les Ulis, France). Normal peripheral blood lymphocytes (PBL) from healthy volunteers were obtained (with their informed consent) following centrifugation on Ficoll gradient. $CD34^+$ hematopoietic progenitor cells were isolated from umbilical cord blood (Bordeaux Maternity, Bordeaux, France) by an immuno-magnetic isolation system (Miltenyi Biotec, Le Perray-en-Yvelines, France), according to the manufacturer's instructions. The purity of the isolated population was assessed by cytofluorometry (Coulter Instrument, Coultronics, Margency, France) after staining with anti-CD34 monoclonal antibody HPCA-2 FITC (Becton Dickinson, Le Pont de Claix, France). Only preparations with >80% $CD34^+$ cells were used in the present work. Total bone marrow-derived mononuclear cells (BM) were obtained from normal graft donors after their informed consent and following centrifugation on Ficoll gradient.

Abbreviations: PBS, phosphate-buffered saline; RES, resveratrol.

Cell cultures

For normal PBL, phytohemagglutinin (PHA, 5 μg/ml, Murex Biotech, Dartford, UK) was added to induce cell proliferation. The various reagents were added at different dilutions as described under results. Following cell cultures during 1–4 days, cells were harvested, washed and counted with trypan blue exclusion. Total bone marrow cells (10 000/ml) or purified $CD34^+$ cells were also plated in 35 mm culture dishes (Falcon, Subra, Toulouse, France) in culture medium containing 30% FCS, 1% bovine serum albumin, 10^{-4} M β-mercaptoethanol, 2 mM L-glutamine and 1% methylcellulose containing colony forming unit (CFU)-inducing cytokines (MethoCult™ GF^+ H4435, Stem Cell Technologies, Meylan, France). The frequency of CFU (>20 cells) was enumerated on day 14.

Cell cycle and caspase analysis

Cell cycle was analyzed as described elsewhere (23). Cells were washed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Pellets were then treated with 200 μl lysis solution (calcium- and magnesium-free PBS, 0.5% Nonidet P-40, 20 μg/ml propidium iodide, 0.2 mg/ml RNase A, 0.5 mM EDTA, pH 7.2; all from Sigma-Aldrich) at room temperature, in the dark for 20 min prior to measurement. DNA analysis was performed by flow cytometry using the System II Coulter program (Coulter Instrument). Activation of caspases was evaluated through DEVDase stimulation and performed as detailed elsewhere (24). Briefly, 7.5×10^4 cells were cultured in triplicates into 96 well plates in the presence or absence of various compounds. After 3 days incubation, the plates were centrifuged, culture supernatant removed and the cells were resuspended in 50 μl of a pH 7.4 buffer containing 10 mM HEPES, 5 mM DTT, 2 mM EDTA, 0.02% saponine, 1 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin (all from Sigma-Aldrich) and 72 μM fluorogenic substrate Ac-DEVD aminomethylcoumarin (UBI Euromedex, Souffelweyerssheim, France). Development of the reaction was read on an automatic spectrofluorometer (Victor 2® Multilabel Counter, Wallac & Perkin Elmer, Akron, OH), at 37°C using l_{exc} = 380 nm and l_{em} = 480 nm. Following this lecture, 200 μl of a 15 μg/ml propidium iodide solution were added to each well and other measures were applied at l_{exc} = 360 nm and l_{em} = 600 nm, to evaluate cell proliferation index. The caspase-3 activity was calculated for 10^5 cells after taking into account the degree of cell proliferation (24). To inhibit caspase activities, we have used Z-VAD-FMK (20–40 μM), a pan-specific caspase inhibitor (21). Leupeptin (20 μM) was also used as inhibitor of caspase-independent proteases (25).

Detection of apoptosis and Fas/FasL expression

To detect apoptosis, externalization of inner membrane phosphatidylserine and DNA content were analyzed by flow cytometry using fluorescein-conjugated annexinV and propidium iodide kit (Immunotech, Marseille, France). At least 10^4 events were analyzed. For FAS/CD95 detection by flow cytometry, we have used FITC-conjugated monoclonal antibody (clone DX2, Pharmingen, San Diego, CA). Detection of Fas ligand (FasL) was performed using 10F2 monoclonal antibody (a kind gift from J.-F.Moreau, Bordeaux 2 University, Bordeaux, France) and FITC-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich).

Detection of cyclins and cyclin-dependent kinase inhibitors

For the detection of cyclins, we have used monoclonal FITC-conjugated antibodies (Pharmingen) as detailed elsewhere (23). Cells were fixed with 80% ethanol at –20°C for at least 2 h incubation for the optimal detection of cyclin E, p21 and p27 by specific monoclonal antibodies (Pharmingen) with simultaneous analysis of DNA content. The cells were then thawed during 30 min, washed, permeabilized with 0.25% Triton X-100 in PBS for 5 min on ice, and washed again with PBS. The cells were then incubated for 2 h at 4°C in the presence of monoclonal antibody to cyclin E, p21 or p27, washed and incubated with a FITC-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich). After washing, the cells were resuspended in 10 mg/ml propidium iodide (Sigma-Aldrich), and 0.1% RNase A (10 U/ml, Roche, Meylan, France) in PBS, and incubated for 20 min before analysis by flow cytometry.

Statistical analysis

Cells were cultured in duplicates or triplicates and each experiment repeated 2–5 times for each cell preparation. Statistical analysis was performed using Systat® software.

Results

RES inhibits the proliferation and induces the apoptosis of various human leukemia cells

RES was tested for its effect on the growth of the following human leukemia cell lines: early myeloid (K562, KCL, HL60), monocytic (U937, THP1), B-lymphoid (WSU-CLL) and T-lymphoid (Jurkat). As shown in Figure 1, RES induced a dose-dependent decrease of leukemia cell number, compared with cells incubated in medium alone. The data also indicated that leukemia cells differ in their sensitivity to RES. As shown in Figure 1 and Table I, Bcr-Abl^+ chronic myelogenous leukemia cell-derived cell lines (K562 and KCL22) (26) and B-chronic lymphoblastic leukemia cell-derived cell line (WSU-CLL) were more resistant to RES compared with other leukemia cells. Surprisingly, promonocytic THP-1 cells showed higher sensitivity to RES, when compared with other leukemia cells. Data in Table I also indicate that the inhibitory doses of RES (IC_{50} = 5–42 μM) were relatively high compared with

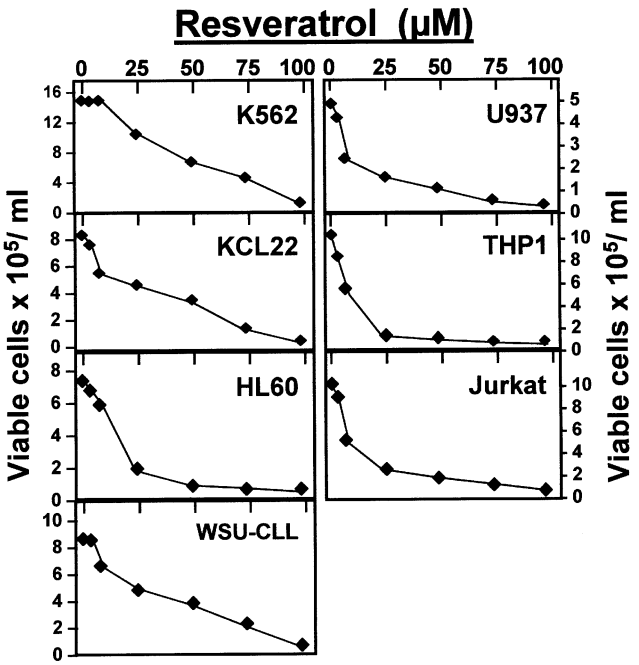

Fig. 1. RES inhibits the proliferation of human myeloid and lymphoid leukemia cells in a dose-dependent manner. Myeloid (K562, KCL22, HL60, U937 and THP-1) or lymphoid (Jurkat and WSU-CLL) leukemia cells were incubated (10^5/ml) during 3 days in the presence of increasing concentrations of RES. Results are mean values from five different experiments (SD < 21%), each done in duplicate.

Table I. Inhibition of various leukemia cell proliferation and induction of their apoptosis by RES

Leukemia cell line	IC_{50}[a] resveratrol (μM)	% Apoptotic cells		Apoptosis induction $P <$[b]
		None	+RES	
K652	42.38	6 ± 3	29 ± 7	0.001
KCL22	38.11	4 ± 2	16 ± 3	0.006
HL60	15.72	4 ± 2	32 ± 5	0.0007
U937	16.09	3 ± 2	12 ± 4	0.008
THP1	5.07	5 ± 3	36 ± 8	0.003
Jurkat	13.42	5 ± 1	20 ± 4	0.001
WSU-CLL	42.76	6 ± 2	21 ± 5	0.01

Various leukemia cells were incubated as in Figure 1 and the RES concentrations achieved 50% growth inhibition (IC_{50}) were given (SD < 21% of mean). The percentage of apoptotic (AnV^+) cells were also measured following 3 days cell incubation with IC_{50} concentrations of RES. Data represent mean ± SD from two distinct experiments for each cell line.
[a]$P < 0.0006$.
[b]Apoptotic cell induction, compared with those incubated without RES.

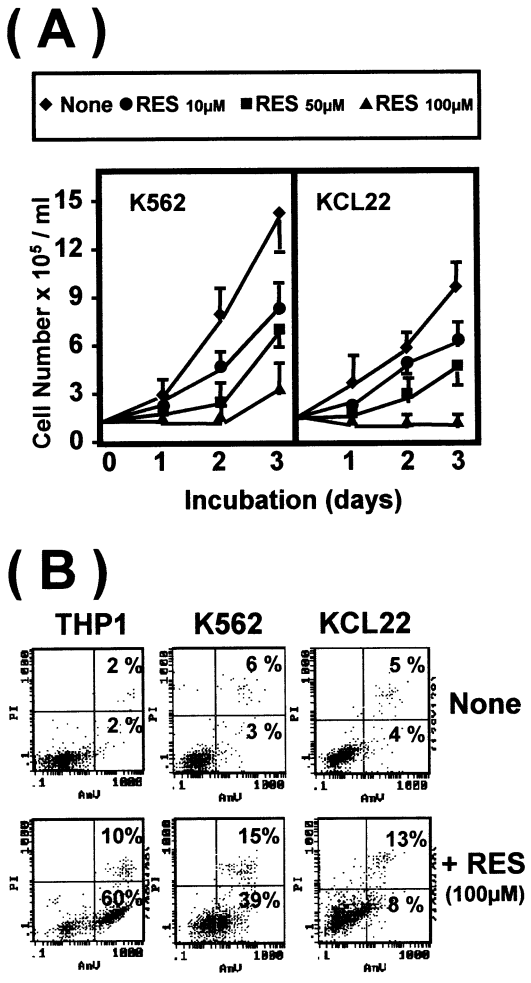

Fig. 2. Inhibition of proliferation by RES correlates with the induction of apoptose in leukemia cells. (**A**) Dose- and time-dependent effects of RES on the proliferation of Bcr-Abl$^+$ leukemia cell lines K562 and KCL22. Following incubation (10^5/ml), leukemia cells were counted every day and results show mean cell number ± SD from two different experiments, each done in duplicate. (**B**) RES induces the apoptosis of human leukemia cells. Cells (10^5/ml) were incubated 3 days in the presence or the absence of RES. They were then tested for their phosphatidylserine externalization through the binding of FITC-conjugated annexinV (AnV) and propidium iodide (PI). Percentage apoptotic cells were given at the top (PI^+AnV^+) and the bottom (PI^-AnV^+) right corners of flow cytometer graphs. Representative data from one experiment, out of three for each cell line.

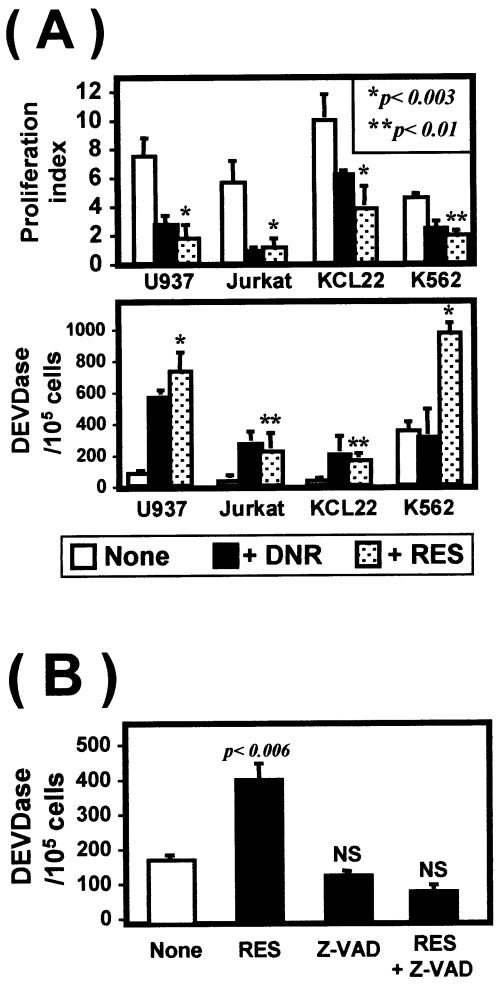

Fig. 3. Constant induction of caspase activity by RES in leukemia cells. DEVD-dependent caspase activity and proliferation index in various leukemia cells (10^5/ml) following their incubation during 3 days with RES (30 μM), compared with DNR (80 nM). (**A**) Caspase activation and cell proliferation index in four distinct leukemia cell lines. Shown are mean ± SD from three experiments. Statistical analysis showed the modulation of the above functions by RES (dotted bars), compared with cells incubated in medium alone (white bars). (**B**) Induction of caspase activity by RES (30 μM) is reversed following cell pre-treatment with Z-VAD-FMK (40 μM). Mean ± SD from three experiments in K562 cells.

their bioavailability in some plants (1,2). Data in Figure 2A clearly show that RES-mediated inhibition of leukemia cell proliferation is dose- and time-dependent. RES can therefore be considered as a constant inhibitory agent for human myeloid or lymphoid leukemia cell growth.

Beside growth inhibition, we asked whether a RES-mediated decrease of leukemia cell numbers was related to apoptosis. Apoptosis has been quantified by the detection of phosphatidylserine externalization, hallmark of membrane apoptosis (27). Data in Table I and Figure 2B show the ability of RES to significantly induce apoptosis in human leukemia cells, although the percentage of apoptotic cells varied regarding the leukemia cell line analyzed. Human BCr-Abl$^+$ cell lines (KCL22 and K562), shown to be more drug-resistant than other leukemia cells (26), also underwent apoptosis in the presence of RES (Figure 2). As suggested previously (16), we investigated the role of Fas/FasL pathway during RES-mediated apoptosis. RES failed to induce significant increases in membrane expression of Fas or FasL antigens (<5% increase) in most leukemia cells tested. In addition, leukemia cell incubation with Fas-neutralizing antibody (clone BMS 140, Bender MedSystem, Ingelheim, Germany) did not modify leukemia cell death, neither did Fas ligation in RES-treated cells (data not shown). These results, obtained with several leukemia cell lines, clearly exclude the role of Fas/FasL pathway during RES-mediated apoptosis.

Induction of caspases by RES in leukemia cells

The ability of RES to DEVD-dependent caspase activities (19,29) in all leukemia cells was investigated. As a positive control, we treated the same cells with daunorubicin (DNR), a cytoreductive agent known to induce caspase activation in human hematopoietic cells (30). As shown in Figure 3A, RES-mediated inhibition of leukemia cell proliferation correlated with the induction of DEVDase activity in all leukemia cells. Of interest, and in contrast to DNR, RES-induced caspase activities in Bcr-Abl$^+$, K562 cells (Figure 3A). Figure 3B

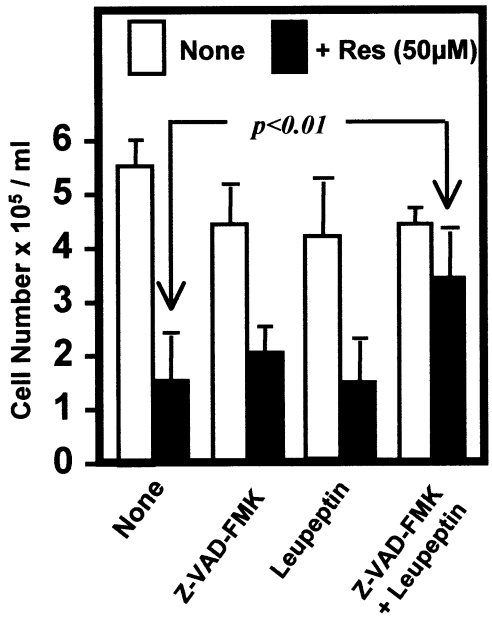

Fig. 4. The role of caspase-dependent and independent proteases during RES-induced apoptosis of leukemia cells. Cells were incubated (10^5/ml) during 3 days with RES (50 µM) alone (None) or in the presence of Z-VAD-FMK (40 µM), leupeptin (20 µM) or both. Only simultaneous addition of both inhibitors reversed RES-mediated growth inhibition. Mean ± SD from three distinct experiments in K562 cell line.

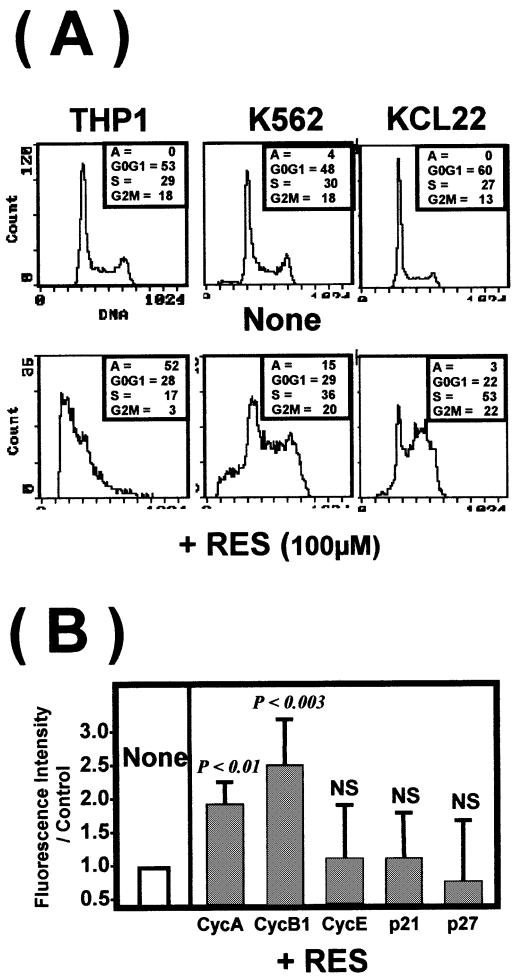

Fig. 5. RES induces cell cycle arrest and modulates cyclin expression in leukemia cells. (**A**) Myeloid leukemia cells (5×10^4/ml) were analyzed 2 days post-incubation with RES and shown are cell cycle graphs with the percentage of cells in each phase mentioned on the top right corner. Percentage of cells with fragmented DNA (A=) was also given. (**B**) Regulation of cyclins (A, B1 and E), p21 and p27 expression by RES (100 µM). Only CycA and CycB1 levels were significantly modulated following 2 day incubation, as compared with cells incubated in medium alone. Shown are mean fluorescence intensities ± SD of two different experiments with 10^4 cells counted (24).

further indicates that cell pre-treatment with Z-VAD-FMK, a pan-specific inhibitor of caspase activity (21), reversed RES-induced activation of caspases in human leukemia cells. To determine the involvement of these enzymes during RES-mediated anti-leukemic activities, cells were pre-treated with Z-VAD-FMK or another protease inhibitor, leupeptin, prior to RES addition in the culture medium. Data in Figure 4 indicate that inhibition of caspases or leupeptin-sensitive proteases (25) alone did not significantly affect RES effects, whereas cell treatment with both inhibitors partially reversed the anti-proliferation and the proapototic activities of RES. The preliminary data suggest that RES used two alternative enzymatic pathways to induce leukemia cell apoptosis.

Regulation of leukemia cell cycle by RES

Cell cycle and cyclin modulations were analyzed following RES addition. Data in Figure 5A show RES-mediated cell cycle modifications in three distinct leukemia cell lines. While THP1 cells rapidly went out of cell cycle through apoptosis, Bcr-Abl$^+$ cells showed clear accumulation in S- and/or G_2/M-phases, correlating with decreased number of cells in the G_1-phase. To determine the exact cell cycle check-point(s) affected by RES, we have analyzed the expression of cyclins and cyclin-dependent kinase inhibitors p21 and p27 (31). Leukemia cells presented a high accumulation of cyclin A and cyclin B1, while the expression of p21 and p27 was not modified (Figure 5B). Cyclin E, expressed in the G_1-phase (31), was not modified following RES addition. Cyclins A and B1 were accumulated in their respective phases, S and G_2, which suggested that cell cycle progression was interrupted at these check-points.

RES inhibits cell growth and induces the apoptosis of normal hematopoietic cells

Various authors (16,18) showed that RES had no significant effect on the survival of non-activated human PBL, and suggested the absence of RES toxicity in normal cells. Cell cycle modifications observed in leukemia cells led us to clarify the hematotoxicity of RES in normal cycling hematopoietic cells. Figure 6 confirms the absence of RES toxicity in non-activated peripheral lymphocytes. In contrast, addition of RES to PHA-activated PBL (mostly T lymphocytes) resulted in a dramatic decrease (Figure 6) in cell proliferation and survival at concentrations (IC_{50} = 8 µM), lower than those required for most human leukemia cells (see Table I). In addition to decreased cell recovery, RES induced the apoptosis in some PBL cells (Figure 6, lower panel). We also investigated the effect of RES on the survival of early hemopoietic cells and their ability to generate colonies *in vitro*. RES significantly ($P < 0.003$) suppressed the generation of colonies from freshly isolated normal human bone marrow cells or CD34$^+$ precursors purified from cord blood (Figure 7) in a similar dose-dependent manner (IC_{50} = 60 µM). In addition to CFU inhibition, RES induced the apoptosis of purified CD34$^+$ hematopoietic cells (Figure 7), few percentage of apoptotic cells were detected in bone marrow cells due to the low percentage of precursor

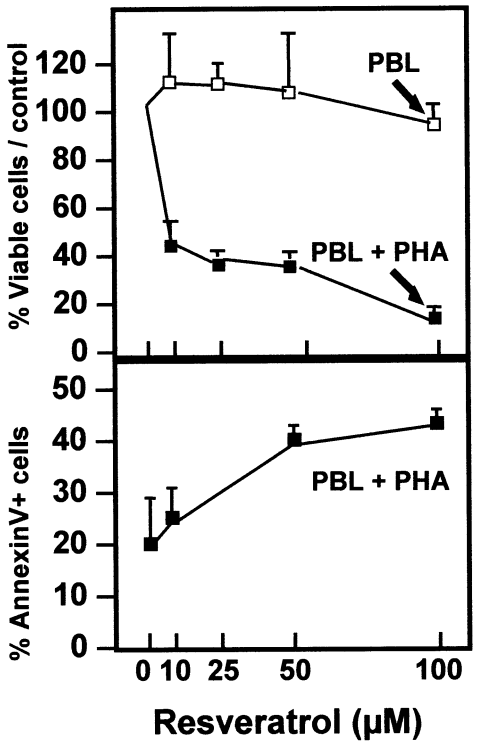

Fig. 6. RES inhibits the growth and induces the apoptosis of activated peripheral lymphocytes in a dose-dependent manner. In contrast to non-activated cells, addition of RES to PHA-activated PBL dramatically decreased their *in vitro* proliferation (upper panel) and slightly increased the percentage of apoptotic cells (lower panel). Shown are mean ± SD from three distinct healthy donor cell preparations, each done in triplicate.

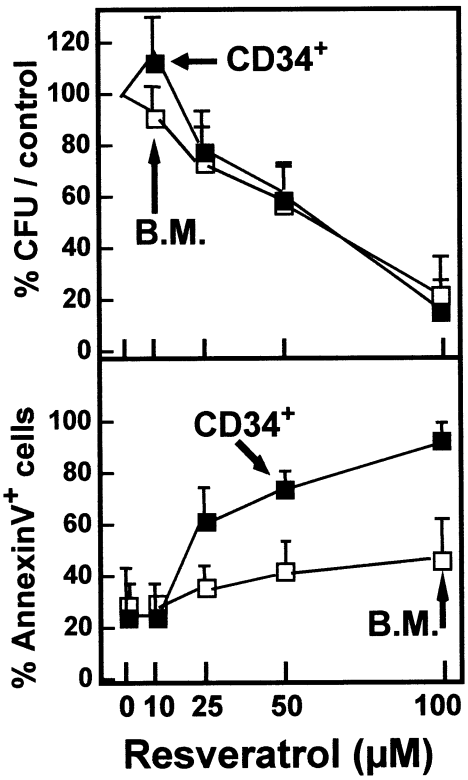

Fig. 7. Decreased *in vitro* cloning capacity and increased apoptosis of early hemopoietic cells by RES. Incubation of total bone marrow-derived mononuclear cells (BM) or early cord blood-derived $CD34^+$ hemopoietic in the presence of RES resulted in a marked decreases in CFU-forming capacities (upper panel). RES also induced the apoptosis (lower panel) of cytokine-activated $CD34^+$ cells and, at lesser extent of BM cells in a dose-dependent manner. Shown are mean ± SD of two distinct cell preparations, each done in duplicate. Cells were incubated 14 or 4 days for CFU and apoptosis assays, respectively.

(cycling) cells in this population. Together, the data revealed the anti-proliferative and the proapoptotic effects of RES in normal hemopoietic cells, although $CD34^+$ cells seem to be more resistant to RES than mature lymphocytes.

Discussion

The results of our study show that RES has anti-proliferative and proapoptotic activities in normal human hematopoietic cells, as already reported in various leukemia cell populations (15–19). RES inhibited the growth of a wide range of lymphoid and myeloid leukemia cells, which suggested a possible common pathway in this phenomenon. In addition to growth inhibition, we have observed RES-induced, dose-dependent apoptosis in all cells tested, although some cell populations (THP-1 and PBL) displayed more sensitivity than others (K562, KCL-22, WSU-CLL and $CD34^+$). Such variability corroborates with the well-known notion fact that leukemia cell lines differ in their *in vivo* or *in vitro* chemosensitivity (28). The mechanism of RES-induced apoptosis was also investigated. The role of the CD95-CD95L system was analyzed because this pathway has been suggested for various drug-induced or immune-mediated clearance of tumor cells (32). The low percentage of $CD95^+$ leukemia cells following RES addition, the failure of CD95 ligation to induce cell death in RES-treated cells, and the inability of CD95 neutralizing antibody to inhibit RES effects make unlikely the involvement of this pathway in RES-derived growth inhibition. In lymphoid leukemia (19,33) and THP-1 (29) cells, authors have also failed to detect any implication of the CD95-CD95L pathway during RES-induced apoptosis. We have also shown that RES induced a dose-dependent increase of caspase activity in all hematopoietic cells tested. However, in contrast to early studies (16,29,33), inhibition of caspase activation alone did not significantly modify RES-mediated growth decrease. We have recently shown that inhibition of not yet identified proteases by leupeptin inhibited caspase-independent proapoptotic pathway in hemopoietic cells (25). By the use of leupeptin, we have shown that RES-mediated effects may be reversed through the use of both Z-VAD-FMK and leupeptin, rather than each inhibitor alone (Figure 4). These results suggest that RES uses both proapoptotic pathways, dependent upon their availability. Dorrie *et al.* (33) have recently suggested the involvement of mitochondrial membrane depolarisation and caspase-9 activation during RES-induced apoptosis of acute lymhpoblastic leukemia cells. Tinhofer *et al*. (34) have further confirmed the involvement of mitochondria during RES-mediated suppression of lymphoid leukemia cell proliferation. Comparing our results, it seems of interest to investigate the relationship between leupeptin-sensitive protease(s) and mitochondria modulation.

The ability of RES to modulate the cell cycle was also investigated. Cyclin gene expression is tightly regulated in a phase-specific manner (31). Cyclin A is synthesized at the onset of the S-phase, reaches a maximum at mitosis and is degraded during the pro-metaphase, shortly prior to cyclin B1

degradation. Cyclin B1 accumulates in the S-phase, reaches a maximum at mitosis, and then is rapidly degraded at the metaphase/anaphase transition. Cyclin E belongs to the 'start' or 'G_1' cyclins, and its accumulation is rate limiting for progression from G_1- to S-phase (31). Cyclin E regulates G_1-phase progression or S-phase commitment in mammalian cells. The onset of cyclin E synthesis begins in the mid-G_1-phase, its expression peaks at the G_1/S-transition, and its stepwise degradation occurs during cell progression through the S-phase. In our hand, RES induced a cell cycle arrest in leukemia cell in the G_2/M-phase, instead of S/G_2- (35) or G_0/G_1-phases (19), corroborating an accumulation of cyclin A and cyclin B1. The expression of cyclin E, p21 and p27 was not modified, in contrast to recent observations in RES-treated bovine endothelial cell cultures (12). Cyclins A and B accumulation in their respective phases (S and G_2) suggested cell cycle arrest at these checkpoints. Subsequent to these events, our data also indicate that hemopoietic cells exhibited elevated cell surface expression of membrane phosphatidyl-serine, indicating loss of phospholipid asymmetry (27). Together, the data point to specific modifications of cell cycle and induction of both nuclear and membrane apoptosis in leukemia cells following their incubation with RES. The ability of RES to decrease NF-κB activity (15) and to inhibit ribonucleotide reductase (36) may constitute possible cell cycle regulators.

As reported previously (16,18), we failed to show RES toxicity on PBL-derived, inactivated normal human cells. However, modification of cell cycle in leukemia cells led us to test the effect of RES on normal human cycling cells: PHA-activated PBL or cytokine-induced proliferation of precursor cells from human bone marrow or purified $CD34^+$ fraction of cord blood cells. $CD34^+$ cells proliferate and generate large amount of hematopoietic cells following their incubation with appropriate growth factors such as stem cell factor, IL-3 and IL-1. The addition of RES to normal cycling hematopoietic cells resulted in significant decreases in cell proliferation and increased apoptosis. PHA-activated cycling PBL (mostly T lymphocytes) showed relatively high sensitivity to RES (IC_{50} <10 μM), whereas bone marrow and cord blood-derived $CD34^+$ cells were slightly less sensitive (IC_{50} = 60 μM) than most leukemia cells (IC_{50} = 5–48 μM). The relatively high toxicity of RES on normal lymphocytes correlated with recent data obtained from normal liver cells (37) and makes it inappropriate as a main cytoreductive therapeutic agent in leukemia. Only *in vitro* depletion of leukemia cells, with absence of normal cell cycle induction may be of potential therapeutic use (18), and may be recommended for multidrug-resistant leukemia cells.

Dramatic inhibition of normal lymphocyte growth in the presence of RES may explain the anti-inflammatory effects of RES (6). Antigen-dependent T-cell expansion is the most common event during immune response, due to their ability to secret various cytokines and to activate macrophage functions (38). In addition to its ability to directly affect macrophages (14,15), RES may therefore induce immuno-suppression by decreasing T-cell expansion and consequent inhibition of T-cell functions. However, to extrapolate our *in vitro* findings to *in vivo* events following RES-containing food consumption, more studies are necessary to determine the stability, half-life and biologically significant concentrations of RES that induce apoptosis *in vivo*. Taken together, our findings strongly suggest that *in vivo* use of RES as cancer chemopreventive or chemotherapeutic agent in humans merits more cautions and further *in vivo* investigations.

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