Cell Death and Differentiation (2000) 7, 834–842

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Resveratrol causes arrest in the S-phase prior to Fas-independent apoptosis in CEM-C7H2 acute leukemia cells

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Received 3.2.00; revised 3.5.00; accepted 11.5.00
Edited by A Finazzi-Agrò

Abstract

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), in the concentration range of 20 μM and above, induced arrest in the S-phase and apoptosis in the T cell-derived T-ALL lymphocytic leukemia cell line CEM-C7H2 which is deficient in functional p53 and p16. Expression of transgenic *p16/INK4A*, which causes arrest in G0/G1, markedly reduced the percentage of apoptotic cells. Antagonist antibodies to Fas or FasL, or constitutive expression of *crmA* did not diminish the extent of resveratrol-induced apoptosis. Furthermore, a caspase-8-negative, Fas-resistant Jurkat cell line was sensitive to resveratrol-induced apoptosis which could be strongly inhibited in the Jurkat as well as in the CEM cell line by z-VAD-fmk and z-IETD-fmk. The almost complete inhibition by z-IETD-fmk and the lack of inhibition by *crmA* suggested caspase-6 to be the essential initiator caspase. Western blots revealed the massive conversion of procaspase-6 to its active form, while caspase-3 and caspase-2 were proteolytically activated to a much lesser extent. *Cell Death and Differentiation* (2000) **7,** 834–842.

Keywords: resveratrol; apoptosis; CEM-C7H2 leukemia cells; caspase; crmA; Fas

Abbreviations: Fas, APO-1/CD95; FasL, Fas ligand; rsFasL, recombinant soluble FasL; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; TBS, TRIS buffered saline; SDS, sodium dodecyl sulfate; crmA, cytokine response modifier A; TNFα, tumor necrosis factor α

Introduction

The polyphenolic phytoalexin resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) became well known as a substance present in grapes and wine, especially in red wine, with pronounced pharmacological effects such as inhibition of cyclooxygenase I, antioxidant and anti-inflammatory activity,[1] inhibition of platelet aggregation and coagulation,[2] modulation of arachidonic acid metabolism,[3] lipid metabolism[4] and inhibition of low density lipoprotein oxidation.[5–7] Based on these properties, it was suggested that resveratrol is a protective agent against cardiovascular diseases and may be responsible for the so-called French paradox,[8,9] namely, the lower frequency of myocardial disease in the population of Southern France, in spite of an intake of fat and cholesterol in amounts constituting a risk factor for cardiovascular disease. As a constituent of root extracts of *Polygonum cuspidatum*[10,11] resveratrol was used in the Japanese and Chinese folk medicine, and was recently identified as a cyclooxygenase I-inhibiting component of the Peruvian root *Cassia quinquangulata*.[1] In assays representing three major stages of carcinogenesis, resveratrol was shown to have anticancerogenic properties.[1] More recently, antiproliferative and apoptosis-inducing effects of resveratrol were observed in certain tumor cell lines such as HL60 leukemia cells, T47D breast carcinoma cells,[12] Yoshida AH-130 rat hepatoma cells,[13] androgen-nonresponsive prostate cancer cells[14] and in a nontransformed bovine pulmonary artery endothelial cell line.[15] Interestingly, resveratrol only marginally reduced the survival of unstimulated peripheral blood lymphocytes.[12] Although the bioassay for cyclooxygenase I inhibition led to purification of the anticancerogenic agent resveratrol, the mechanisms of anticancerogenicity as well as apoptosis triggering of resveratrol remain unknown. Besides inhibition of cyclooxygenase I, recently discovered antiproliferative effects of resveratrol, such as inhibition of ribonucleotide reductase,[16] inhibition of SV40 DNA replication in CV-1 monkey kidney cells,[17] and other antiproliferative effects,[18–20] may also contribute to the anticancerogenic and apoptosis-inducing properties of resveratrol.

In a previous study it was reported that in HL60 leukemia cells and in T47D breast carcinoma cells resveratrol-induced apoptosis was specifically Fas-dependent.[12] In the CEM-C7H2 leukemia cell line of the present study, however, blocking of either Fas or Fas ligand signaling with antagonistic antibodies or constitutive expression of the cowpox serpin *crmA* had no influence on apoptosis induction by resveratrol. Furthermore, a caspase-8-deficient (Fas- and TNFα-resistant) Jurkat mutant cell line was sensitive to apoptosis triggering by resveratrol which – as in CEM-C7H2 cells – could be almost completely inhibited by z-IETD-fmk.

Cell cycle analysis revealed that resveratrol causes arrest in the S-phase in a concentration-dependent manner,

with the arrest occurring at an earlier time point of the S-phase at higher concentrations of resveratrol. Activation of transgenic *p16/INK4A* caused in CEM-C7H2 cells (which are p16-deficient) an arrest in G0/G1, and this resulted in a marked reduction in the extent of resveratrol-induced apoptosis. Based on these observations it is suggested that the primary event in resveratrol-induced apoptosis in CEM-C7H2 cells is the arrest of DNA replication. The lack of inhibition by *crmA* and the strong inhibition by z-IETD-fmk was indicative of caspase-6 being essential for triggering resveratrol-induced apoptosis in CEM-C7H2 leukemia cells. Western blotting demonstrated the massive presence of caspase-6 and detectable amounts of the active forms of caspase-2 and caspase-3 in the early phase of resveratrol-induced apoptosis.

Results

Time- and dose-dependence of resveratrol-induced apoptosis in CEM-C7H2 leukemia cells

Figure 1 shows that resveratrol is a potent apoptosis inducer in CEM-C7H2 cells. Apoptosis detection and quantification was performed by FACS analysis of propidium iodide stained nuclei[21] (Figure 1A) and annexin V binding to cells[22] (Figure 1B). Nuclear DNA fragmentation could be observed with 20 μM resveratrol after 48 h and with 60 μM after 24 h. The application of 100 μM resveratrol caused almost 40% apoptosis after 24 h and about 70% after 48 h with only a minor further increase at 72 h. The picture in Figure 1B – using annexin V binding for apoptosis detection – was similar, but apoptosis was detected earlier, i.e. 24 h after application of 40 μM resveratrol and 48 h after application of 20 μM resveratrol.

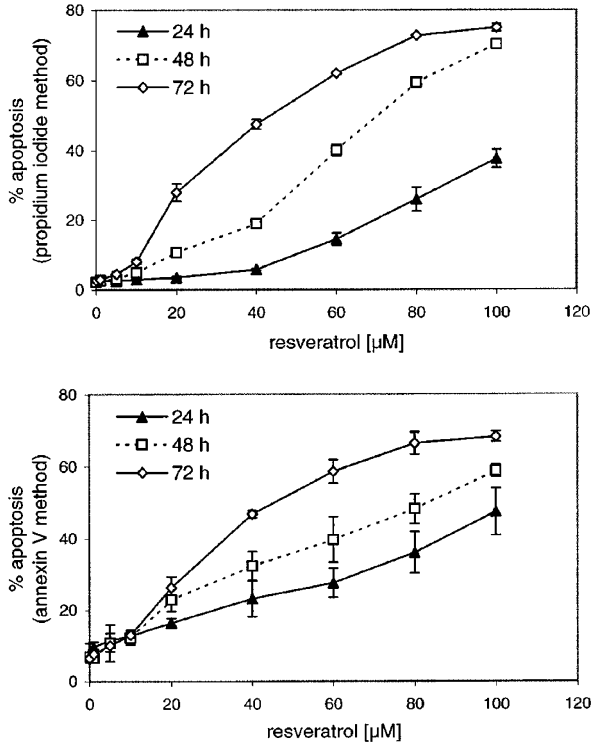

Figure 1 Time- and dose-dependence of resveratrol-induced apoptosis. (**A**) The percentage of apoptosis was determined by FACS analysis of propidium iodide-stained nuclei of CEM-C7H2 cells. (**B**) The percentage of apoptosis was determined by FACS analysis of Annexin V binding to CEM-C7H2 cells. Data of a representative experiment are shown as means $\pm$ S.D. of triplicates. Each experiment (in triplicate) was performed at least three times

Effects of resveratrol on the cell cycle

Next we tested the effects of resveratrol on the cell cycle. The cell cycle analysis by FACS (Figure 2), which is based on the same procedure as the apoptosis assay by nuclear propidium iodide fluorescence (see Materials and Methods section), revealed an accumulation of cells in the S-phase. Figure 2 shows the concentration dependence of the cell cycle distribution of CEM-C7H2 cells after 24 h of incubation at the given concentrations of resveratrol. Accumulation of cells in S-phase can be seen after application of 10 μM resveratrol, which keeps increasing with 20 μM resveratrol; at 40 μM, the accumulated cells in early S-phase are very close to the G1 peak, at 100 μM they are so close to the G1 peak that the G1/S boundary of S-phase cannot be distinguished from G1. The DNA profiles are, however, difficult to interpret when cell death is initiated in a cell cycle phase other than G0/G1. The question has to be addressed whether Figure 2 shows arrest in the S-phase or rather nuclei with reduced DNA contents after apoptosis induction in G2, which are mistaken for S-phase arrested ones. In support of an arrest in S-phase it should be pointed out: (i) no increase of the G2 peak as a prelude to resveratrol-induced apoptosis was observed in CEM-C7H2 cells; (ii) there is a fairly good agreement in the

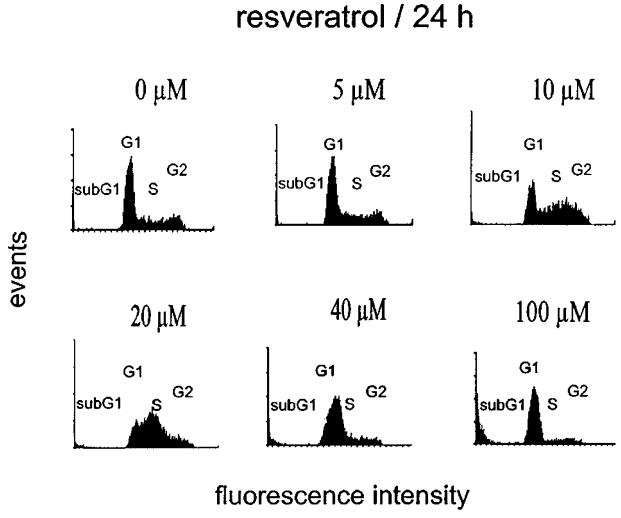

Figure 2 Effects of resveratrol on the cell cycle. Resveratrol treatment of CEM-C7H2 cells results in an accumulation of cells in the S-phase and a decrease of the percentage of cells in G2/M. For cell cycle analysis, propidium iodide-stained nuclei were analyzed by FACS in the same manner as for quantification of apoptosis; however, the fluorescence intensity is plotted on a linear scale. The distribution of CEM-C7H2 cells as related to the resveratrol concentration is shown in histograms. The sub-G1 region of apoptotic cells is indicated to the left of the G1 peak. The experiment was performed at least three times

percentages of apoptotic cells, when comparing the data of propidium iodide fluorescence (in which nuclei in the sub-G1 region are considered as apoptotic) (Figure 1A) with those of annexin V binding (phosphatidylserine surface exposure) (Figure 1B); (iii) after 24 h with 10 and 20 μM resveratrol the DNA profile shows substantial arrest in S-phase (Figure 2) but a very low level of apoptosis is measured with annexin V; even with 40 and 100 μM resveratrol, after 24 h, masses of nuclei are accumulated at the G1/S boundary, but only about 20 and 40% apoptosis, respectively, is measured by annexin V binding. Taken together, the data of Figures 1 and 2 suggest that in the course of resveratrol-induced apoptosis, arrest in the S-phase comes first (Figure 2), followed by apoptosis detectable by annexin V (Figure 1B) and subsequently also by propidium iodide (Figure 1A).

Resveratrol-induced apoptosis is abrogated by *p16/INK4A*-mediated arrest in G0/G1

Since it was not possible to distinguish between G1 and very early S-phase with the above-described cell cycle analysis, and further, according to our postulate apoptosis is triggered after arrest in the S-phase, we wanted to see whether resveratrol is able to induce apoptosis in cells which have been arrested in G1. For this purpose, we used subclones of CEM-C7H2 (which itself is p16-deficient) stably transfected with the *p16/INK4A*-gene under the control of the doxycycline 'tet-on' system. In these constructs, the transgenic *p16/INK4A* gene[23] can be activated by doxycycline, which causes arrest in G0/G1 without inducing apoptosis.[24]Resveratrol-induced apoptosis was measured under conditions of activated transgenic *p16/INK4A* gene *versus* the nonactivated *p16/INK4A* construct, i.e. in the presence and absence of doxycycline in the culture media. If apoptosis by resveratrol is triggered by a specific event which is restricted to the S-phase then apoptosis induction should be prevented by the arrest in G0/G1. Figure 3 shows that this indeed is the case. The induction of the transgenic *p16/INK4A* gene led to a marked reduction in the percentage of resveratrol-induced apoptosis with a decrease from 38 to 16%. Cells were grown in the presence or absence of 200 ng/ml doxycycline for 24 h and then 100 μM resveratrol was added for a further 24 h incubation. Three clones stably transfected with *p16/INK4A* were tested and with all three the partial prevention of resveratrol-induced apoptosis could be observed as a result of overexpression of *p16/INK4A*. The CEM-C7H2 cell line which is investigated in the present study is deficient not only in p16 but also in functional p53. Thus, resveratrol-induced apoptosis in this cell line is operative in the absence of functional p53 and p16.

Blocking of Fas or the Fas ligand or constitutive expression of *crmA* does not affect resveratrol-induced apoptosis

Next we tested whether resveratrol exerts any apoptosis signaling via Fas or Fas ligand. We were prompted to do this in view of a previous study on resveratrol-induced apoptosis in HL-60 cells and T47D breast carcinoma cells[12] in which resveratrol-induced apoptosis was correlated with upregulation of surface expression of Fas and Fas ligand and was sensitive to inhibitory antibodies against Fas or FasL. Figure 4A shows, however, that in CEM-C7H2 leukemia cells, blocking of either Fas or Fas ligand with antagonistic mabs (ZB-4, NOK-1) did not diminish the extent of resveratrol-induced apoptosis, which thus in this cell line has to take place without direct interaction between Fas and the Fas ligand. We also measured the expression levels of Fas and Fas ligand after incubation with 100 μM resveratrol for 24 h and observed unchanged levels (Figure 4B). Thus, in CEM-C7H2 leukemia cells the mechanism of resveratrol-induced apoptosis involves neither the upregulation of Fas ligand or Fas nor the activation of Fas by Fas ligand.

To confirm further the lack of involvement of the Fas-receptor-complex in resveratrol-induced apoptosis, we tested the effect of the cowpox serpin *crmA* (which blocks Fas- and TNFα-mediated apoptosis) in stably transfected CEM-C7H2 cells which constitutively express *crmA*.[25] Figure 5 shows that in this cell line, CH-11, an agonistic antibody for Fas, is unable to induce apoptosis, whereas resveratrol-induced apoptosis occurs to the same extent as in the parental cell line. *CrmA* is an inhibitor of group I (i.e. of caspase-1, caspase-4 and caspase-5) and group III caspases with the exception of caspase-6 (i.e. of caspase-8, caspase-9 and caspase-10).[26–28] Thus, with the exception of caspase-6, these caspases cannot play an essential role in resveratrol-induced apoptosis in CEM-C7H2 cells.

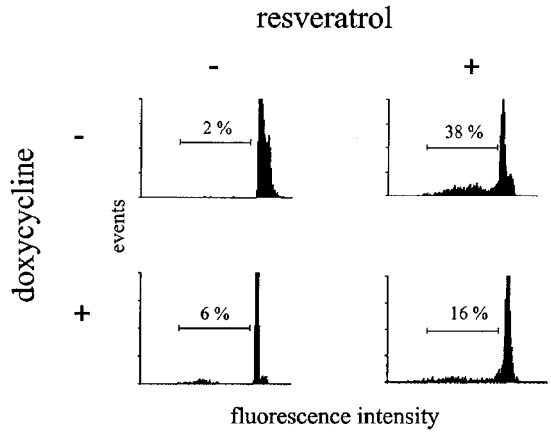

Figure 3 Abrogation of resveratrol-induced apoptosis by expression of transgenic *p16/INK4A*. The C7H2^{tetp16} cell line 1E10 is a *p16/INK4A*-transfected derivative of C7H2-2C8. The promoter of the transfected *p16/INK4A* becomes activated by doxycycline using the 'tet-on' system. Before addition of resveratrol, cells were treated with doxycycline for 24 h and subsequent resveratrol treatment (100 μM) was for 24 h. Fluorescence intensity is plotted on a logarithmic scale. Because of the logarithmic plot, the G1 and G2 peaks are very close to each other. The conspicuously highest peak is the G1 peak. Very near to it to the right with some overlap is the G2/M peak. The G2/M peak is highest in control cells treated neither with resveratrol nor with doxycycline (p16-negative condition without apoptosis inducer). To the left of the G1 peak is the sub-G1 peak representative of apoptotic cells. The percentage of cells in this sub-G1 peak is indicated with numbers in the four panels of the graph. A representative experiment is shown. Experiments were performed at least three times. A similar partial prevention of resveratrol-induced apoptosis was also observed with the other two *p16/INK4A*-transfected cell lines 1D2 and 6E2

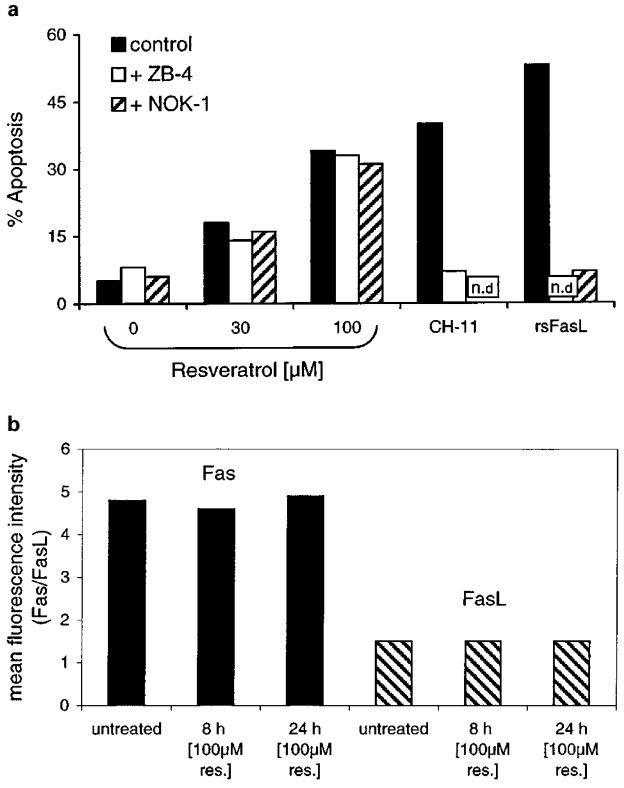

Figure 4 Resveratrol-induced apoptosis is insensitive to inhibition of Fas/FasL-mediated apoptosis. (**A**) Resveratrol-induced apoptosis in CEM-C7H2 cells is not blocked by inhibitory antibodies against Fas (ZB-4) or Fas ligand (NOK-1). There is no significant inhibition of resveratrol-induced apoptosis by the inhibitory antibodies, this is shown at apoptosis induction with 30 and 100 μM resveratrol for 48 h. Note that apoptosis induced either by the agonistic antibody CH-11 or by the recombinant soluble Fas ligand rsFasL is completely blocked by ZB-4 or NOK-1 (5 μg/ml each). A representative experiment is shown. (**B**) For detection of surface Fas and FasL expression, FITC-labeled murine anti-human Fas (clone UB2, Immunotech, Marseille, France) and FITC-labeled anti-human FasL (clone H11, Alexis, Läufelfingen, Switzerland) were used. Briefly, 0.5×10^6 cells were stained with the respective specific mabs (1 μg) or an isotype-matched negative control mab for 30 min at 4°C, washed and immediately analyzed by flow cytometry. A representative experiment is shown. Mean fluorescence intensity is the quotient of specific anti Fas/FasL fluorescence and the fluorescence of an unspecific isotype control. Under the conditions of resveratrol-induced apoptosis, there is no change in the level of either Fas or Fas ligand; (n.d., not done)

A caspase-8-negative Jurkat cell line is sensitive to resveratrol-induced apoptosis

We also tested a caspase-8-negative mutant Jurkat cell line, which expresses neither caspase-8a nor caspase 8b,[29] for sensitivity to resveratrol-induced apoptosis. Figure 6 shows that this caspase-8-negative Jurkat mutant cell line is resistant to Fas apoptosis but sensitive to resveratrol-induced apoptosis. Thus, in the investigated CEM and Jurkat cell lines the triggering of apoptosis by resveratrol occurred without the participation of FasL, Fas and caspase-8. Moreover, in the Jurkat cell line (Figure 6) as well as in the CEM cell line (Figure 7) resveratrol-induced apoptosis was inhibited by z-IETD-fmk, which is consistent with an essential role of caspase-6 in apoptosis induction in both cell lines.

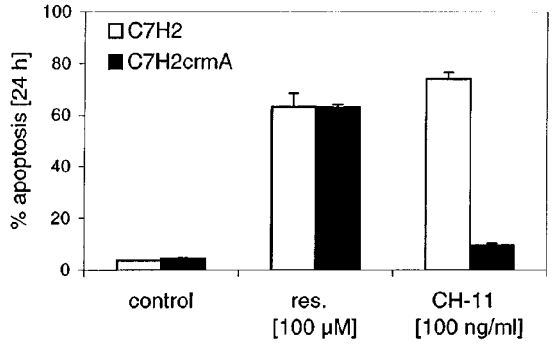

Figure 5 Resveratrol-induced apoptosis is insensitive to crmA. Resveratrol-induced apoptosis (100 μM) is not inhibited in the constitutively *crmA* expressing cell line C7H2^{crmA}-2E8. Note the almost complete inhibition of Fas-induced apoptosis (agonistic anti-Fas mab CH-11, 100 ng/ml) and at the same time the complete lack of effect on resveratrol-induced apoptosis. Means $\pm$ S.D. of a representative experiment, performed in triplicate, are shown

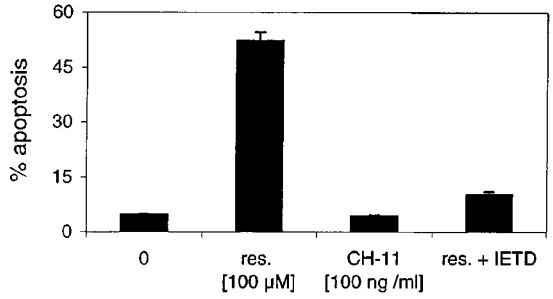

Figure 6 Resveratrol induces apoptosis in the absence of caspase-8. The caspase-8-negative mutant Jurkat cell line[27] is resistant to apoptosis induction by the agonistic Fas mab CH-11 but sensitive to resveratrol; z-IETD-fmk inhibits resveratrol-induced apoptosis in the caspase-8 deficient Jurkat cells. Apoptosis induction was for 24 h. Means $\pm$ S.D. of a representative experiment, performed in triplicate, are shown

Sensitivity to inhibitory peptides and detection of caspases by immunoblotting

In order to get more information on the pathway of resveratrol-induced apoptosis in CEM-C7H2 cells, various peptide inhibitors of caspases were tested. Figure 7 shows the results of these experiments. z-VAD-fmk, a general caspase inhibitor (but a weak inhibitor of caspase-2[26,27]) and z-IETD-fmk, an inhibitor of group III caspases (caspases-6, -8, -9 and -10)[26] proved to be the two most effective inhibitors of resveratrol-induced apoptosis. The other caspase inhibitors tested, namely, z-YVAD-fmk, a less efficient inhibitor of group III caspases,[26] z-VEID-fmk, an inhibitor with some preference for caspase-6[30] and z-DEVD-fmk, an inhibitor of caspase-3, -7 and -8[27] exerted partial inhibition of resveratrol-induced apoptosis within the investigated time periods of 24 and 48 h. Since z-IETD-fmk, an inhibitor of caspase-6 but not of caspase 3, caused almost complete apoptosis inhibition, the activation of caspase-6 has to occur upstream of and as a prerequisite for the subsequent activation of caspase-3 (DFF45/ICAD-protease).[31]

In order to further test the conclusions of the inhibition experiments, we probed for the presence of the active

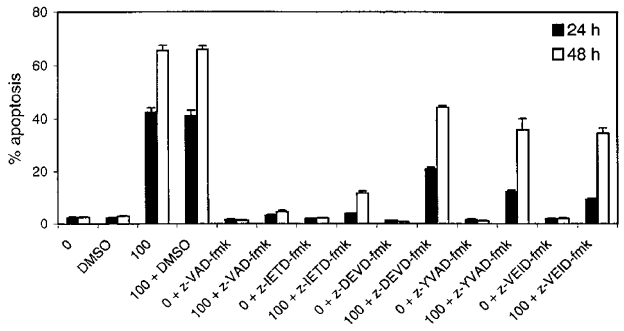

Figure 7 The effects of peptide inhibitors of caspases on resveratrol-induced apoptosis. The percentage of apoptic cells was determined 24 h (black bars) and 48 h (open bars) after induction with resveratrol. DMSO was used as solvent for resveratrol as well as for the caspase inhibitor peptides. The numbers 0 and 100 refer to μM resveratrol added. At zero μM resveratrol, 10 μl vehicle (DMSO) was added. Data are means $\pm$ S.D. of triplicates of a representative experiment. Experiments (in triplicate) were performed at least three times

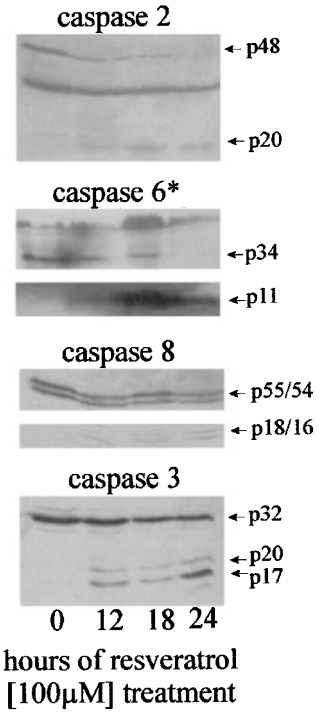

Figure 8 Immunoblot analysis of caspase activation. The Western blots show that the active forms of caspase-2, caspase-6 and caspase-3 are present before caspase-8 becomes detectable. Note the virtually complete conversion of procaspase-6 (p34) to its active form (p11). Apoptosis induction with resveratrol was for 12, 18 and 24 h. Lysates of whole cells were applied to SDS-polyacrylamide gel electrophoresis. Western blotting was performed with antibodies against the human caspases as described in Materials and Methods. Because of the low molecular mass of the active form of caspase-6, two membranes were applied in Western blotting. Procaspase-6 (p34) is blotted to the first membrane; the active form of caspase-6 (p11) is revealed on the second membrane

forms of several caspases by immunoblot analysis. Figure 8 shows that the active forms of caspase-6, -3 and -2 could be detected already 12 and 18 h after apoptosis induction. While at the time points 12, 18 and 24 h procaspase-6 (p34) was converted as good as completely to its active form, the level of procaspase-3 (p32) was hardly diminished, only a minor portion of it being proteolytically activated. After 24 h the active forms of caspase-8 also became detectable. The strong inhibition with z-IETD-fmk (together with the lack of inhibition by crmA) indicated that caspase-6 plays a key role in this apoptosis pathway. The immunoblots of Figure 8 are consistent with this supposition, as caspase-6 was virtually completely converted to its active form, while the other examined caspases remained to a large extent in their inactive precursor forms even 24 and 48 h after apoptosis induction.

Discussion

In the present study it is shown that resveratrol is a potent apoptosis inducer in CEM-C7H2 cells. Until recently the main interest in resveratrol focused on its beneficial effects on the cardiovascular system. It was only in 1997 that cancerprotective effects of resveratrol became known and since then a few reports have emerged on the apoptosis-inducing capacity of resveratrol in tumor cells.[12–14] Little is known about the cell type-specificity and the mechanism of resveratrol-induced apoptosis. In a previous study it was shown that in the investigated HL60 cell line, and also in T47D breast carcinoma cells, resveratrol-induced apoptosis was strictly Fas/FasL-dependent.[12] In contrast to this, in the T cell-derived lymphocytic CEM-C7H2 cell line investigated in the present study, apoptosis induction by resveratrol took place with undiminished efficiency regardless of whether Fas or Fas ligand was blocked by inhibitory antibodies or whether *crmA* was expressed constitutively. It should be emphasized that *crmA* is a very potent inhibitor of Fas- and/or TNFα-induced apoptosis.[32,33] Since *crmA* is an inhibitor of group I (i.e. of caspase-1, caspase-4 and caspase-5) and group III caspases with the exception of caspase-6 (i.e. of caspase-8, -9 and -10),[26,27] resveratrol-induced apoptosis in CEM-C7H2 cannot be triggered via caspase-8 or by some deviation from this route via caspase-9 or caspase-10. This is further underlined by the fact that a Fas- and TNFα-resistant caspase-8-negative Jurkat cell line was found to be sensitive for apoptosis induction by resveratrol (Figure 6).

The tight cell cycle dependence of apoptosis sensitivity – with its abrogation by *p16/INK4A*-mediated arrest in G0/G1 – strongly suggested that it is the nucleus where the primary apoptosis signal is produced by resveratrol. Resveratrol caused arrest of the cell cycle in the S-phase and cell cycle analysis revealed that the higher the concentration of resveratrol, the earlier is the point in S-phase at which cells are arrested. The cell cycle analysis of the present study is based on DNA content, as measured by FACS analysis of propidium iodide stained nuclei. One may argue that the DNA profiles depicted in the histograms of Figure 2 do not represent nuclei of S-phase arrested cells but rather those of apoptotic G2 arrested ones with DNA contents reduced to various extents; if so, the sub-G1 peak would be a gross underestimate in quantifying apoptotic cells. However, the following data of the present

study appear to be inconsistent with such an interpretation: (i) an increase of the G2 peak as a result of resveratrol treatment was never observed in CEM-C7H2 cells; and arrest in G0/G1 by transgenic p16/INK4A expression protected these cells from resveratrol-induced apoptosis; (ii) the percentages of apoptotic cells determined as the sub-G1 peak of propidium iodide stained nuclei[21] (Figure 1A) are in good agreement with those determined by annexin V membrane staining of whole cells[22] (Figure 1B), with the exception of the earliest time point measured (24 h), as annexin V staining detects apoptosis already at an earlier stage, before the onset of nuclear fragmentation; (iii) a massive arrest in S-phase was observed at the lower resveratrol concentrations of 10 and 20 μM after 24 h (Figure 2) but at the same time very little apoptosis induction was detected with annexin V staining of whole cells (Figure 1B). It should be further added that, when performing FACS analysis, parallel to nuclear propidium iodide fluorescence we also measured light scattering by the nuclei under investigation. Dot-analysis of propidium iodide fluorescence *versus* forward light scatter revealed that the apoptotic nuclei appeared to be shrunk – before any DNA loss could be detected by propidium iodide fluorescence analysis – and once DNA fragmentation started they dropped to the sub-G1 peak level without producing a spectrum of intermediate DNA contents indistinguishable from different stages of the S-phase (the data of the light scattering analysis are not shown). From our observations it can be concluded that either at a certain stage of apoptosis, nuclear DNA is very rapidly degraded to reach the DNA content of the sub-G1 region, or/and condensation of the fragmented nuclear DNA causes a sudden drop in its stainability by propidium iodide. According to the applied technique of Nicoletti *et al*,[21] propidium staining of nuclei is performed in a hypotonic buffer in the presence of 0.1% Triton X-100, which results in the rupture of the plasma membrane, the release of small DNA fragments form the nuclei and at the same time a better penetration of nuclei by propidium iodide is enabled.

Thus, our observations are consistent with the interpretation that resveratrol in CEM-C7H2 cells causes an arrest in S-phase, which precedes the onset of DNA fragmentation characteristic of later-stage apoptotic cells. It should be also noted that in a Jurkat cell line, resistant to apoptosis induction by resveratrol, we observed arrest in S-phase without subsequent apoptosis induction as a result of resveratol treatment (unpublished observations). In HL60 promyelocytic leukemia cells, an arrest in S-phase occurred in the absence of a G2/M peak at resveratrol concentrations (30 μM) which did not induce apoptosis but caused induction of differentiation.[34] In that study it was also shown that under these conditions of resveratrol treatment the G1 cell cycle engine remains undisturbed in HL60 cells.[34] Furthermore, at concentrations which did not induce apoptosis, resveratrol suppressed proliferation of bovine pulmonary artery endothelial cells by perturbing progression through S-phase and G2.[15]

The question arises as to how resveratrol causes arrest in S-phase. It was reported that resveratrol is an inhibitor of ribonucleotid reductase in murine lymphoblastic leukemia cells[16] and an inhibitor of proliferation in K562 human erythroleukemia cells and P815 murine mastocytoma cells[16] and of SV40 replication in CV-1 monkey kidney cells.[17] If such an inhibitory activity holds true for CEM-C7H2 cells as well, it would explain the arrest in S-phase and subsequent induction of apoptosis out of the S-phase. Consistent with our hypothesis is also a report according to which resveratrol is capable of binding to DNA and in the presence of Cu^{2+} to cleave DNA.[35] Thus, we would like to suggest that the arrested replication machinery with stalled replication forks provides the primary signal which in certain cell types results in apoptosis induction. However, in the investigated cell line the mechanism cannot be a generic DNA strandbreak-induced apoptosis, as it was shown in our previous studies that strandbreak-induced apoptosis in CEM-C7H2 cells triggered by X-rays[36] or doxorubicin[37] caused accumulation of cells and apoptosis in the G2-phase of the cell cycle. Nonetheless, it is conceivable that resveratrol-induced apoptosis is triggered by a different type of DNA lesion.

The possibility that resveratrol, a hydrophobic triphenol, partitions into the plasma membrane and leads to an active conformation of a death receptor on the cytosolic side can be disregarded because of the above-mentioned cell cycle dependence of resveratrol sensitivity. For the same reason, a direct effect of resveratrol on the mitochondria also appears unlikely as the primary apoptosis triggering mechanism.

The inhibition experiments with the peptide inhibitors and the constitutively expressed *crmA* strongly suggested that caspase-6 is the essential initiator caspase of the investigated apoptosis pathway (almost complete inhibition by z-IETD-fmk and lack of inhibition by *crmA*). z-IETD-fmk caused this strong inhibition of resveratrol-induced apoptosis not only in CEM-C7H2 cells (Figure 7) but also in the caspase-8-negative mutant Jurkat cell line (Figure 6).

In Figure 8 the immunoblots show the active forms of caspase-6 and -3 and -2 to be present 12, 18 and 24 h after apoptosis induction. Only a rather small portion of procaspase-2 and -3 became activated, while a massive conversion of procaspase-6 to its active form can be seen. As outlined in a recent review,[26] the activation of caspase-6 occurs before the activation of caspase-3, -2 and -7. Thus caspase-6 (lamin A protease)[38,39] is not only an effector but also a key initiator caspase.[28] According to this molecular ordering of caspases,[26] caspase-6 should be responsible in the investigated apoptosis pathway for the activation of caspase-3 (DFF45/ICAD protease),[31] which then leads to the observed DNA-fragmentation (propidium iodide assay). Furthermore, caspase-7 and positive feedback loops involving activated caspases[40] can be expected to participate in resveratrol-induced cell death in CEM-C7H2 cells. Here we have shown that the investigated apoptosis pathway is operative without the crmA-sensitive caspases (caspase-1, -4, -5, -8, -9 and -10), is strongly inhibited by z-VAD and z-IETD, and massive conversion of procaspase-6 to its active form occurs,

There are two major sites in the cell from which death signals can be initiated, namely, the various death-inducing signal complexes of the plasma membrane, and the

apoptosis machinery of the mitochondria. The present study demonstrates that in CEM-C7H2 cells resveratrol-induced apoptosis is clearly Fas- and TNFα-independent. It remains to be clarified in further investigations whether the apoptosis pathway of resveratrol in this leukemia cell line is linked to a death receptor other than Fas and the TNFα-receptor or is, alternatively, entirely independent of the plasma membrane and linked to the mitochondria.

Since resveratrol was reported to be an inhibitor of cyclo-oxygenase I[1] and cyclooxygenase II[41] and various cyclooxygenase inhibitors can function as cell type-specific apoptosis inducers, we tested sulindac, indomethacin and sulindac sulfide as apoptosis-inducers in CEM-C7H2 cells (data not shown). Sulindac (200 μM) did not induce any apoptosis, while 100 μM indomethacin (which inhibits cyclooxygenase I more effectively than resveratrol)[1] and 100 μM sulindac sulfide were, compared to resveratrol, poor apoptosis inducers (data not shown).

It should also be noted that, based on its structure, resveratrol, a triphenolic stilben, may act as a phytoestrogen. Indeed, it was reported that resveratrol acts as an estrogen agonist[42–44] or antagonist[45] and binds to the estrogen receptor.[46] Thus, we cannot rule out the involvement of a putative receptor in the apoptosis-inducing effect of resveratrol.

Our working hypothesis is that it is the inhibition of DNA-replication by which the primary apoptosis-inducing signal of resveratrol arises in CEM-C7H2 cells. It remains to be clarified by further studies how this event is linked to activation of caspases. It should also be explored in which other cell lines apoptosis is induced by resveratrol independent of Fas and the TNFα-receptor.

With all the caution called for in drawing conclusions from *in vitro* cell line experiments regarding the responses of cancer cells *in vivo*, the dietary component resveratrol, based on its selective actions, may be claimed to be of relevance in prevention as well as therapy of cancer. Resveratrol was only marginally toxic to peripheral blood lymphocytes at concentrations which induced apoptosis in HL60 promyelocytic leukemia cells.[12] The fact that in an HL60 cell line and T47D breast carcinoma cells resveratrol triggered apoptosis via Fas/FasL,[12] whereas in CEM-C7H2 leukemia cells resveratrol-induced apoptosis is Fas and TNFα-independent, shows that in various resveratrol-sensitive cell types different pathways of apoptosis induction can be operative. In another study of HL60 cells it was shown that resveratrol – at non-apoptotic concentrations – is a potent differentiation-inducer after causing arrest in S-phase.[34] Resveratrol protected K562 erythroleukemia cells against oxidative stress-induced apoptosis as well as against certain other unrelated types of apoptosis, such as apoptosis induced by cisplatin, TGF β1 or 5-hydroxyeicosatetraenoic acid.[47] Interestingly, in that study it was also determined that inhibition of prostaglandin synthesis was correlated with the observed anti-apoptotic effects of resveratrol in K562 leukemia cells. This appears to be in utter contradiction to investigations with other cell types in which the inhibition of prostaglandin biosynthesis was correlated with pro-apoptotic effects of various drugs; for instance, cyclooxygenase II expression made intestinal epithelial cells refractory to butyrate-induced apoptosis, and inhibition of cyclooxygenase II made them sensitive.[48]

Since tumor cells develop strategies to escape Fas-mediated apoptosis, the cell type-specific triggering of Fas-independent apoptosis in a p53- and p16- negative background is a desirable property of a potential new therapeutic agent.[49–51] In the present study we have shown that in CEM-C7H2 lymphocytic leukemia cells resveratrol triggered arrest in the S-phase and subsequently Fas-independent apoptosis in the absence of functional p53 and p16 genes. A recent study, however, reported that resveratrol-induced apoptosis occurred only in wild-type p53, but not in p53-deficient mouse fibroblasts.[52] These seemingly contradictory reports of studies on resveratrol performed with different cell types stand in need of explanation. In several previous studies with other apoptosis inducers, it was also observed that the p53 dependence of apoptosis pathways can be strongly cell type-specific.[53,54] The delicate balance between oxidant/antioxidant properties of a given compound might explain its pro- as well as anti-apoptotic effects. Thus, the cell type specificity of redox micro-environments in different cell types may be decisive for an oxidant or anti-oxidant effect of resveratrol. Further, such differences in the redox status could determine the impact of prostaglandin synthesis on the balance between apoptosis/survival or apoptosis/differentiation in a given cell type. Whatever the mechanism behind the cell type specificity of resveratrol, in its contrasting effects lies its potential to be a highly selective drug as differentiation- or apoptosis-inducer against certain types of tumors.

Materials and Methods

Materials

Resveratrol was provided under a Material Transfer Agreement by Pharmascience Inc. (Montreal, Quebec, Canada). Stock solution (100 mM) of resveratrol was prepared in DMSO and kept at -20°C. Inhibitory peptides: benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk), benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (z-IETD-fmk), benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethylketone (z-YVAD-fmk) and benzyloxycarbonyl-Val-Glu-Ile-Asp-fluoromethylketone (z-VEID-fmk) were obtained from Enzyme Systems Products, (Dublin, CA, USA) and kept as 10 mM stock solutions in DMSO at -20°C. The agonistic Fas mab CH-11 (mouse IgM) and the antagonistic Fas mab Z-B4 (mouse IgG1) were from Immunotech (Marseille, France); the antagonistic FasL mab NOK-1 (mouse IgG1) was from Pharmingen (San Diego, CA, USA); rsFasL was from Alexis (Läufelfingen, Switzerland). TNFα was from Eubio (Vienna, Austria).

Cell lines and culture conditions

CEM-C7H2[55] is a glucocorticoid-sensitive subline of CCRF-CEM-C7.[56] C7H2-2C8 is a subclone of the CEM-C7H2 cell line stably transfected with the reverse tetracycline-controlled transcriptional transactivator, rtTA.[57,58] The cell lines C7H2^{tetp16}-1E10, 6E2, and 1D2[24] are derivatives of the C7H2-2C8 cell line that are stably

transfected with *p16/INK4A* under the control of the tetracycline-sensitive transcriptional transactivator. Expression is controlled by rtTA, i.e., it is induced by tetracycline ('tet-on' system). For induction of *p16/INK4A*, 200 ng/ml doxycycline was added to the media 24 h prior to treatment with the apoptosis inducer. The generation of C7H2 sublines stably transfected with constructs allowing constitutive expression of the cowpox virus caspase inhibitor *crmA* (cell lines $C7H2^{crmA}$-2E8, 2G10, 2H10)[25] has been described. The caspase-8-negative mutant Jurkat cell line was a generous gift of Dr. Peter Juo, Department of Cell Biology, Harvard Medical School; it was isolated from the Fas apoptosis-sensitive Jurkat subclone A3 after three cycles of exposure to the frameshifting mutagen ICR191.[29] For all the cell lines investigated, tissue culture medium was RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. All cell lines were grown in 5% CO_2 and saturated humidity at 37°C.

Apoptosis assay

Detection and/or quantification of apoptosis was performed by FACS analysis of nuclear propidium iodide fluorescence[21] or annexin V-FITC binding to cells.[22] The dose- and time-dependence of resveratrol-induced apoptosis is shown with the propidium iodide (Figure 1A) as well as the annexin V methods (Figure 1B). In all other figures of this study the percentage of apoptosis was determined by FACS analysis of propidium iodide stained nuclei as described previously.[21,59] Briefly, 5×10^5 cells were permeabilized and stained with 750 μl propidium iodide (50 μg/ml in 0.1% Triton X-100/0.1% sodium citrate) and subjected to apoptosis analysis in a FACScan (FL-2 channel, Becton Dickinson, San Jose, CA, USA; equipped with an argon laser). Based on propidium iodide staining, cells in the sub-G1 marker window were considered to be apoptotic. Parallel to nuclear propidium iodide fluorescence, light scattering was also measured. According to the light scattering analysis, apoptotic nuclei are recognized as being smaller (lower forward scatter values) and more granulated (higher sideward scatter values). Cell debris and small particles were excluded from the analysis by forward/sideward scatter criteria as described.[60] Annexin V binding[33] was determined using the TACS Annexin V-FITC kit (Trevigen, Gaithersburg, MD, USA), as described by the manufacturer. Approximately 2.5×10^5 cells were washed, incubated with FITC-labeled annexin V and analyzed on a FACScan. Annexin V does not bind to viable cells but binds to apoptotic cells already at an early stage of apoptosis.

Cell cycle analysis

For cell cycle analyses, the propidium iodide method of Nicoletti *et al*,[21] was used as described above for determination of apoptosis by propidium iodide staining of nuclei, except that the fluorescence intensity was plotted on a linear rather than a logarithmic scale.

Western blotting

For immunoblotting, cells were washed in PBS, suspended in sample loading buffer (2% SDS, 10 mM Tris, pH 7.4), boiled for 1 min and sonicated. Equal protein amounts of cell lysates were separated in SDS–polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After blocking nonspecific binding sites for 1 h with 5% nonfat milk in TTBS (TBS with 0.1% Tween 20), the membranes were incubated for 2 h at room temperature with antibodies against caspase-2, caspase-3, caspase-6 (Pharmingen, San Diego, CA, USA) or caspase-8 (Upstate Biotechnology, Lake Placid, NY, USA). The antibodies were dissolved in blocking buffer (TTBS with 5% nonfat milk). The membranes were washed three times in TTBS and incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit antibodies (Amersham, Les Ulis, France), after which they were washed again three times in TTBS. The immunoblots were revealed using an enhanced chemiluminescence detection kit (ECL Western blotting detection reagents RPN 2106) (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Acknowledgements

This work was supported by grants from the Austrian Science Fund (SFB-F204), the Österreichische Krebshilfe-Krebsgesellschaft Tirol and the Province of Tyrol. We are indebted to Ms. Rajam Csordas-Iyer for critical reading and editorial assistance.

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