Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway

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Resveratrol, a plant constituent enriched in the skin of grapes, is one of the most promising agents for the prevention of cancer. However, the mechanism of the anti-carcinogenic activity of resveratrol is not well understood. Here we offer a possible explanation of its anti-cancer effect. Resveratrol suppresses tumor promoter-induced cell transformation and markedly induces apoptosis, transactivation of p53 activity and expression of p53 protein in the same cell line and at the same dosage. Also, resveratrol-induced apoptosis occurs only in cells expressing wild-type p53 (p53+/+), but not in p53-deficient (p53−/−) cells, while there is no difference in apoptosis induction between normal lymphoblasts and sphingomyelinasel-deficient cell lines. These results demonstrate for the first time that resveratrol induces apoptosis through activation of p53 activity, suggesting that its anti-tumor activity may occur through the induction of apoptosis.

Introduction

Apoptosis, or programmed cell death, has been characterized as a fundamental cellular activity occurring under a wide range of physiological and pathological conditions (1–6). It plays an essential role as a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells or excess cells that have been improperly induced to divide by a mitotic stimulus (7–9). Growing evidence from both in vitro and in vivo studies demonstrates that suppression of apoptosis is involved in tumor promotion by chemical agents (10–13). Many agents, such as phenobarbital, peroxisome proliferators, cyproterone acetate and dichloroacetic acid, have been shown to suppress the basal rate of apoptosis in the liver (10–12). The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibited apoptosis of C3H-10T1/2 cells induced by ionizing radiation, β-radiation or acute serum deprivation (13). In the rat hepatocyte model of carcinogenesis, the formation of neoplastic liver foci can be enhanced if a single exposure to an initiating carcinogen is followed by chronic treatment of animals with a tumor promoter such as phenobarbital (14,15). These effects appear to result predominantly from a decreased level of apoptosis within the liver foci since continuous exposure to phenobarbital does not produce a persistent increase in cell proliferation. Interestingly, cessation of phenobarbital treatment causes the regression of foci due to stimulation of apoptosis (16). Therefore, suppression of apoptosis may be a feature of tumor promotion by chemical carcinogens. Indeed, many chemopreventive agents may act through the induction of apoptosis as a mechanism of anti-carcinogens (11,17).

Resveratrol, a phytoalexin which has been found in at least 72 plant species, especially in grape skin (18–20), is one of the most promising chemopreventive agents for cancer. It was shown to have an anti-carcinogenic effect in a two-stage mouse skin cancer model (21). Although the inhibition of tumor formation by resveratrol is thought to occur through blocking effects at the stages of tumor initiation, promotion and progression, the molecular mechanism of its anti-tumor activity is not clear. Here we used a mouse JB6 epidermal cell line, a well-developed cell culture model for studying tumor promotion (22–27), to investigate the molecular mechanism of the chemopreventive effect of resveratrol.

Materials and methods

Reagents

Resveratrol was from Sigma (St Louis, MO); dimethyl sulfoxide (DMSO) was from Pierce (Rockville, IL); protein G plus/protein A–agarose, monoclonal mouse IgG against p53, polyclonal rabbit IgG against p53, epidermal growth factor (EGF) was from Collaborative Research (Bedford, MA); luciferase assay substrate was from Promega (Madison, WI); fetal bovine serum (FBS), Eagle’s minimal essential medium (MEM), Dulbecco’s modified Eagle’s medium (DMEM), RPMI1640 and basal medium Eagle (BME) were from BioWhittaker (Walkersville, MD).

Cell culture

JB6 P+ mouse epidermal cell line C1 41 and its stable p53–luciferase reporter plasmid transfect, C1 41 p53 cells, were cultured in monolayers at 37°C, under 5% CO2 using MEM containing 5% fetal calf serum, 2 mM L-glutamine and 25 µg/ml gentamycin (22,23,28). Epstein–Barr virus (EBV)-transformed normal human lymphoblast cell lines, JY, or Niemann–Pick disease lymphoblast line MS1418 (29,30), cells were maintained in a mixture of RPMI1640 and DMEM (1:1 v/v) containing 15% FBS, 2 mM L-glutamine and 25 mg/ml gentamycin. Normal embryo fibroblasts (p53+/+) or p53-deficient embryofibroblasts (p53−/−) were cultured in DMEM with 10% FBS, 2 mM L-glutamine and 25 mg/ml gentamycin (31).

Anchorage-independent transformation assay

Inhibition of resveratrol on TPA- or EGF-induced cell transformation was investigated in JB6 C1 41 cells. Samples of 1×105 cells were exposed to TPA (10 ng/ml) or EGF (10 ng/ml) with or without different concentrations of resveratrol in 1 ml 0.33% BME agar containing 10% FBS over 3.5 ml 0.5% BME agar medium containing 10% FBS. Resveratrol was dissolved in DMSO. The same concentration of DMSO (<0.1%) was used in the control group. The cultures were maintained in a 37°C, 5% CO2 incubator for 14–21 days and the cell colonies were scored by the methods described (22–26).

Assay for p53 activity

Confluent monolayers of C1 41 p53 cells were trypsinized and 8×103 viable cells, suspended in 100 µl MEM with 5% FBS, were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO2. After 12–24 h, cells were starved by culturing them in MEM with 0.1% FBS for 12 h. The cells were exposed for 24 h to different concentrations of resveratrol for p53 induction. Resveratrol was dissolved in DMSO. The

Abbreviations: BME, basal medium Eagle; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; EBV, Epstein–Barr virus; EGF, epidermal growth factor; FBS, fetal bovine serum; MEM, Eagle’s minimal essential medium; SMase, sphingomyelinase; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Fig. 1. Inhibition of EGF- or TPA-induced JB6 C1 41 cell transformation by resveratrol. Aliquots of 10^4 JB6 C1 41 cells were exposed simultaneously to either TPA (10 ng/ml) or EGF (10 ng/ml) with or without different concentrations of resveratrol in 0.33% BME agar containing 10% FBS over 0.5% BME agar containing 10% FBS. Cell colonies were scored after 14 days incubation at 37°C in 5% CO2. The results are expressed as described previously (22). Each bar indicates the mean and standard deviation of assays from triplicate experiments.

Fig. 2. Induction of apoptosis by resveratrol in JB6 C1 41 cells. (a) Subconfluent (80–90%) monolayer JB6 C1 41 cells in 100 mm dishes were subjected to different concentrations of resveratrol for 24 h. Then, both detached and attached cells were harvested for DNA fragmentation assay as described in Materials and methods; (b–e) subconfluent (80–90%) monolayer JB6 C1 41 cells on microscope slides were treated with medium containing 0.1% DMSO as control (b) or 2.5 (c), 10 (d) or 40 µM (e) resveratrol, respectively, for 24 h. Then, the cells were fixed on slides and DNA fragment end labeling assays were performed as described in the Klenow FragEL DNA fragmentation detection kit from Oncogene.

Results and discussion

From animal investigations, resveratrol has been shown to have a potent protective effect against the development of tumors (21). However, the molecular mechanism of the anti-carcinogenic effect of resveratrol is not clear. To investigate the molecular basis of its anti-tumor promotion effect, we first tried to establish a cell culture model for studying anti-tumor promotion effects by using JB6 C1 41 cells. As shown in...
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Figure 3. Resveratrol induces apoptosis in both JY and MS1418 cells. SMase-deficient MS1418 cells or normal lymphoblast JY cells were treated with resveratrol for 24 h. Then, (a) the cells were harvested and DNA fragmentation assays were performed as described in Materials and methods; (b–e) the cells were fixed and attached to microscope slides, the DNA fragment end labeling assays were done as described in the Klenow FragEL DNA fragmentation detection kit from Oncogene: (b) JY cell control; (c) JY cells treated with 10 µM resveratrol; (d) MS1418 cell control; (e) MS1418 cells treated with 10 µM resveratrol.

Figure 1, resveratrol inhibits tumor promoter (TPA or EGF)-induced JB6 C1 41 cell transformation in a dose-dependent manner in the range 2.5–40 µM (Figure 1). This concentration range is relevant regarding possible biological effects of resveratrol in the daily consumption of grape beverages (18–20,32,33).

Inhibition of apoptosis is one mechanism of tumor formation and many chemopreventive agents may act through the induction of apoptosis to block the carcinogenic process (11–17,34,35). We therefore hypothesized that the induction of apoptosis may be involved in the anti-carcinogenic effect of resveratrol. To test this, we treated JB6 C1 41 cells with different concentrations of resveratrol. The results from both DNA fragmentation assays and a DNA fragment end labeling assay show that treatment of cells with resveratrol induced apoptosis in C1 41 cells in the same dose range which inhibited cell transformation (Figure 2). These results support our notion that the cancer preventive effect of resveratrol may occur through the induction of apoptosis.

Apoptosis results from activation of a pre-programmed pathway of biochemical events leading to cell death (34,35). A large body of evidence indicates that apoptosis may represent a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells that have improperly been induced to divide by factors such as carcinogens (7–9). It is known that a number of factors and pathways can lead to apoptosis (36–39). Ceramide and/or sphingomyelinase (SMase) activity is required for many agents such as TNF-α, interferon or γ-ionizing radiation to induce apoptosis (7–9,29,30,36–39). To determine whether ceramide/SMase is involved in resveratrol-induced apoptosis, we used a SMase-deficient lymphoblast line, MS1418 and a normal lymphoblast control cell line, JY (29, 30). As shown in Figure 3, resveratrol induced apoptosis in both cell lines (Figure 3). These data rule out the involvement of ceramide/SMase in resveratrol-induced apoptosis.

Normal p53 function was shown to be crucial in the induction of apoptosis in human and murine cells following DNA damage (40,41). This notion was further supported by the findings that p53 is the most commonly mutated tumor suppressor gene and lack of p53 expression or function is associated with an increased risk of tumor formation (42–44). Apoptosis of thymocytes and intestinal crypt cells following irradiation was almost completely blocked in p53-deficient mice (41,45,46) and incidence of spontaneous tumors in p53−/− mice was high and occurred rapidly (47). To investigate the
Fig. 4. Induction of p53 transactivation and gene expression by resveratrol. For the p53 transactivation assay, JB6, C1 41, PG13-luciferase (PG13-luc) stable transfectant cells were used. C1 41 p53 cells suspended in 5% FBS MEM were added to each well of 96-well plates and cultured overnight. The cells were treated with resveratrol at the concentration indicated for 24 h (a) or with 20 µM resveratrol for different times (b). The p53 activity was determined by luciferase activity assay (28). The results are presented as relative p53 activity (28). (c) For detection of p53 gene expression, a subconfluent monolayer of C1 41 cells in 100 mm dishes was subjected to 10 µM resveratrol for the times indicated. The p53 protein was immunoprecipitated using specific p53 antibody (Ab1; Oncogene) and the levels of p53 protein were measured by western blot using p53 antibody (Ab10; Oncogene) and a chemiluminescent detection system.

Fig. 5. Resveratrol induces apoptosis in p53+/+ fibroblasts, but not in p53−/−. Subconfluent monolayers of p53+/+ or p53−/− cells on microscope slides were treated with 10 µM resveratrol for 24 h. Then, the cells were fixed on slides and DNA fragment and labeling assays were performed as described in the Klenow FragEL™ DNA fragmentation detection kit from Oncogene. (a) p53+/+ cell control; (b) p53+/+ cells treated with 10 µM resveratrol; (c) p53−/− cell control; (d) p53−/− cells treated with 10 µM resveratrol.
possible role of p53 in the induction of apoptosis by resveratrol, we analyzed the influence of resveratrol on p53-dependent transcription activity and p53 protein expression in PG13-luciferase JB6 cell transfectants (28). We found that resveratrol markedly activates p53-dependent transcription activity in a dose-dependent manner (Figure 4a). p53-dependent transcription activity reached maximum induction at 24 h after cells were exposed to resveratrol (Figure 4b). Treatment of cells with resveratrol led to an elevated level of p53 protein (Figure 4c). These results indicate that induction of p53 protein by resveratrol is at least partially responsible for increasing p53-dependent transcriptional activity, suggesting that increased p53 protein and p53-dependent transcriptional activity may be associated with resveratrol-induced apoptosis. To obtain direct evidence for the involvement of p53 in resveratrol-induced apoptosis by resveratrol, we used two fibroblast cell lines, p53+/+ and p53−/−, which were derived from mouse embryos containing either wild-type p53 (p53+/+) or were p53-deficient (p53−/−) as reported previously (31). The results show that p53−/− fibroblasts failed to respond to resveratrol with apoptosis induction, while apoptosis was observed in p53+/+ cells treated with resveratrol (Figure 5). These results provide definitive evidence for the requirement of p53 in resveratrol-induced apoptosis.

In summary, we demonstrate here that resveratrol induces apoptosis in the same concentration range that inhibits cell transformation. The resveratrol-induced apoptosis occurs through a p53-dependent, ceramide/SMase-independent pathway. We suggest that the induction of apoptosis is a possible explanation for the anti-cancer effect of resveratrol.

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