

# Sensitization for Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Induced Apoptosis by the Chemopreventive Agent Resveratrol

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## ABSTRACT

Survivin is a member of the inhibitor of apoptosis proteins that is expressed at high levels in most human cancers and may facilitate evasion from apoptosis and aberrant mitotic progression. Naturally occurring dietary compounds such as resveratrol have gained considerable attention as cancer chemopreventive agents. Here, we discovered a novel function of the chemopreventive agent resveratrol: resveratrol is a potent sensitizer of tumor cells for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through p53-independent induction of p21 and p21-mediated cell cycle arrest associated with survivin depletion. Concomitant analysis of cell cycle, survivin expression, and apoptosis revealed that resveratrol-induced G<sub>1</sub> arrest was associated with down-regulation of survivin expression and sensitization for TRAIL-induced apoptosis. Accordingly, G<sub>1</sub> arrest using the cell cycle inhibitor mimosine or induced by p21 overexpression reduced survivin expression and sensitized cells for TRAIL treatment. Likewise, resveratrol-mediated cell cycle arrest followed by survivin depletion and sensitization for TRAIL was impaired in p21-deficient cells. Also, down-regulation of survivin using survivin antisense oligonucleotides sensitized cells for TRAIL-induced apoptosis. Importantly, resveratrol sensitized various tumor cell lines, but not normal human fibroblasts, for apoptosis induced by death receptor ligation or anticancer drugs. Thus, this combined sensitizer (resveratrol)/inducer (e.g., TRAIL) strategy may be a novel approach to enhance the efficacy of TRAIL-based therapies in a variety of human cancers.

## INTRODUCTION

Despite aggressive therapies, resistance of many tumors to current treatment protocols still constitutes a major problem in cancer therapy (1). Thus, current attempts to improve cancer survival will have to include strategies that specifically target tumor cell resistance. The aggressive cancer cell phenotype is the result of a variety of genetic and epigenetic alterations leading to deregulation of intracellular signaling pathways (2).

Apoptosis, the cell's intrinsic death program, plays a crucial role in the regulation of tissue homeostasis, and an imbalance between cell death and proliferation may result in tumor formation (3). Also, killing of tumor cells by diverse cytotoxic approaches such as anticancer drugs,  $\gamma$ -irradiation, suicide genes, or immunotherapy is predominantly mediated through induction of apoptosis in tumor cells (4–10). Apoptosis pathways may be initiated through different entry sites, such as death receptors or mitochondria, resulting in activation of effector caspases (5). Stimulation of death receptors of the tumor necrosis factor receptor superfamily such as CD95 (APO-1/Fas) or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors results in caspase-8 activation, which can directly translate into cleavage of downstream effector caspases (11–14). The mito-

chondrial pathway is initiated by the release of apoptogenic factors such as cytochrome *c*, apoptosis-inducing factor (AIF), Smac (second mitochondria-derived activator of caspase)/DIABLO, or endonuclease G from the mitochondrial intermembrane space. The release of cytochrome *c* into the cytosol triggers caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex. Smac/DIABLO promotes caspase activation through neutralizing the inhibitory effects to inhibitor of apoptosis proteins (IAPs), whereas AIF and endonuclease G cause apoptotic DNA degradation (15, 16).

Targeting death receptors to trigger apoptosis in tumor cells is an attractive concept for cancer therapy (11). To this end, TRAIL appears to be a relatively safe and promising death ligand for clinical application. TRAIL has been shown to exhibit potent tumoricidal activity in a variety of human cancer cell lines *in vitro* and also *in vivo* in several xenograft models with minimal or no toxicity to nonmalignant human cells (17). However, many tumors remain resistant to treatment with TRAIL, and this resistance may be caused by deregulated expression of antiapoptotic molecules (17–19). IAPs such as survivin are expressed at high levels in many tumors including neuroblastoma and have been associated with refractory disease and poor prognosis (20, 21). IAPs block apoptosis at the core of the apoptotic machinery by inhibiting effector caspases. Thus, therapeutic modulation of IAPs could target a key control point in deciding cell fate and therapy resistance, e.g., resistance to TRAIL (22, 23).

In recent years, naturally occurring antioxidant compounds present in diet and beverages such as resveratrol have gained considerable attention because of their beneficial effects on health as cancer chemopreventive or cardioprotective agents (24). Resveratrol (3,4',5-trihydroxystilbene) is a polyphenol synthesized by plants and is present in dietary items such as grapes and red wine (25). Also, resveratrol has been reported to exert antitumor activity (25). In the search for new strategies to overcome resistance of tumors, we tested the anti-tumor activity of resveratrol on human tumor cell lines in the present study. We discovered a novel function of resveratrol by showing for the first time that resveratrol is a potent sensitizer for TRAIL-induced apoptosis in a variety of human tumor cell lines by a mechanism that involves p21-mediated cell cycle arrest and survivin depletion.

## MATERIALS AND METHODS

**Cell Culture.** Neuroblastoma (SHEP, GIMEN, and LAN5), medulloblastoma (PSFK), glioblastoma (U373MG and A172), melanoma (SK-Mel and Colo38), pancreatic carcinoma (MiaPaCa2), prostate carcinoma (LNCaP), or breast carcinoma (MCF7) were maintained in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) as described previously (8). p53<sup>+/+</sup>, p53<sup>-/-</sup>, p21<sup>+/+</sup>, or p21<sup>-/-</sup> HCT116 colon carcinoma cells (kindly provided by Dr. B. Vogelstein; Baltimore, MD) were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated FCS, 10 mM HEPES (Biochrom, Berlin, Germany), and 2 mM L-glutamine (Biochrom). Human fibroblasts (PromoCell, Heidelberg, Germany) were maintained according to the manufacturer's instructions. Cells (0.5 × 10<sup>5</sup> cells/ml) were cultured in 96-, 24-, or 6-well plates or in 75-cm<sup>2</sup> flasks (Falcon, Heidelberg, Germany).

**Determination of Apoptosis, Clonogenic Growth, and Caspase Activity.** Cells were incubated with resveratrol, doxorubicin, VP16, cisplatin, mimosine, thymidine, nocodazole, cycloheximide (all from Sigma, Deisenhofen, Germany), TRAIL (PeproTech, Rocky Hill, NJ), anti-APO1 (CD95) mono-

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clonal antibody (8), pifithrin- $\alpha$  (Alexis, Grünberg, Germany), and benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone [(zVAD-fmk) (Bachem, Heidelberg, Germany)] at the indicated concentrations and times. Apoptosis was assessed by fluorescence-activated cell-sorting (FACS) analysis of DNA fragmentation of propidium iodide-stained nuclei or by annexin V staining (Roche Diagnostics, Mannheim, Germany) as described previously (8). Clonogenic growth was assessed by crystal violet staining as described previously (9). Caspase activity was determined using rabbit anti-active caspase-3 polyclonal antibody (BD Bioscience) and flow cytometry as described previously (26). Mean and SD of triplicates are shown, and similar results were obtained in three independent experiments unless otherwise indicated.

**Cell Cycle Analysis.** Analysis of cell cycle and/or apoptosis was performed by flow cytometric staining of permeabilized cells with propidium iodide for DNA content and with annexin-FITC for apoptosis as described previously (8). Briefly,  $10^6$  cells were stained with annexin-FITC for 20 min at 4°C, fixed with 4% paraformaldehyde (Sigma) for 20 min at 4°C, permeabilized with digitonin (Sigma; 50  $\mu$ g/ml) for 5 min at room temperature, and stained with propidium iodide (15  $\mu$ g/ml) for 15 min at 37°C in the presence of RNase A (Sigma; 20  $\mu$ g/ml).

**Intracellular Staining.** Intracellular staining of survivin was performed by flow cytometry as described previously (27) using rabbit polyclonal anti-survivin antibody (R&D Systems, Wiesbaden-Nordenstadt, Germany), isotype control, or FITC-labeled antirabbit immunoglobulin (BD Bioscience, Heidelberg, Germany).

**Antisense Oligonucleotides.** To inhibit survivin expression, phosphothiorate antisense oligonucleotides against survivin expression and mismatch control oligonucleotides (Thermo Hybaid, Ulm, Germany) with published sequences were used as described previously (28).

**Reverse Transcription-PCR.** Expression of *survivin* or *p21* mRNA was assessed by reverse transcription-PCR as described previously (29, 30) using primers p21-F (5'-AGGATCCATGTCAGAACCGGCTGG-3'), p21-R (5'-CAGGATCCTGTGGGCGGATTAGGGCT-3'), survivin-F (5'-GCATGGG-TGCCCGACGTTG-3'), and survivin-R (5'-GCTCCGCCAGAGGCCT-CAA-3'; Thermo Hybaid). Survivin primers detect *survivin* and its isoforms *survivin-2B* and *survivin- $\Delta$ Ex3* (29). Expression of *glyceraldehyde-3-phosphate dehydrogenase* mRNA was used to control for equal gel loading.

**Western Blot Analysis.** Western blot analysis was performed as described previously (8), using mouse anti-caspase-8 monoclonal antibody C15 (1:10 dilution of hybridoma supernatant; kindly provided by P. Kramer), mouse anti-caspase-3 monoclonal antibody (1:1000; BD Biosciences), mouse anti-caspase-7 monoclonal antibody (1:1000; BD Biosciences), rabbit anti-caspase-9 polyclonal antibody (1:1000; PharMingen, San Diego, CA), mouse anti-X-linked inhibitor of apoptosis protein (XIAP) monoclonal antibody (1:1000; H62120; BD Biosciences), mouse anti-green fluorescence protein (GFP) monoclonal antibody (1:1000; BD Biosciences), mouse anti-p53 monoclonal antibody (1:1000; BD Biosciences; PharMingen), rabbit anti-survivin polyclonal antibody (1:1000; R&D Systems), rabbit anti-Bid polyclonal antibody (1:1000; R&D Systems), rabbit anti-p21 polyclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-cellular inhibitor of apoptosis protein (cIAP2) polyclonal antibody (1:1000; Santa Cruz Biotechnology), rabbit anti-cellular inhibitor of apoptosis protein (cIAP1) polyclonal antibody (1:1000; R&D Systems), mouse anti-Bcl-2 monoclonal antibody (1:1000; Santa Cruz Biotechnology), rabbit anti-Bak polyclonal antibody (1:1000; Santa Cruz Biotechnology), rabbit anti-Bcl-X<sub>L</sub> polyclonal antibody (1:1000; Santa Cruz Biotechnology), rabbit anti-Bax polyclonal antibody (1:1000; Upstate Biotechnology, Lake Placid, NY), rabbit anti-FLIP polyclonal antibody (1:1000; PharMingen), mouse anti-TRAIL monoclonal antibody (1:1000; PharMingen), mouse anti-Fas-associated death domain protein (FADD) monoclonal antibody (1:1000; BD Biosciences), mouse anti-DFF45/DFF40 monoclonal antibody (1:1000; BD Biosciences), mouse anti-heat shock protein 70 (HSP70) monoclonal antibody (1:1000; Stressgen Biotechnologies Corp., Victoria, Canada), mouse anti-cyclin D1 monoclonal antibody (1:1000; PharMingen), or mouse anti- $\beta$ -actin monoclonal antibody (1:5000; Sigma) followed by goat antimouse IgG or goat antirabbit IgG (1:5000; Santa Cruz Biotechnology). Enhanced chemiluminescence (ECL; Amersham Pharmacia, Freiburg, Germany) was used for detection. Expression of  $\beta$ -actin was used to control for equal gel loading. Densitometric analyses of scanned immunoblotting images were performed using NIH Image software.

**Transfection Experiments.** SHEP neuroblastoma cells were transiently transfected by LipofectAMINE (Life Technologies, Inc.) with pGEM-4Z vector or pGEM-p21 (Promega, Madison, WI) as described previously (9). For cycloheximide block experiments, SHEP neuroblastoma cells were transiently transfected with a vector containing GFP-tagged survivin by LipofectAMINE as described previously (31). Sixteen h after transfection, cells were treated or not treated with 100  $\mu$ M resveratrol in the presence of 2  $\mu$ g/ml cycloheximide to prevent further expression of transfected plasmids, harvested at 0 or 24 h after cycloheximide block, and analyzed for expression of GFP-containing fusion proteins by Western blotting with an antibody to GFP.

**Promoter Analysis.** SHEP neuroblastoma cells were transiently transfected with a vector containing the survivin promoter ligated to the promoterless firefly luciferase gene (pGL2-survivin; Promega) together with cytomegalovirus-*Renilla* luciferase (Promega) as described previously (32). Sixteen h after transfection, cells were treated with 100  $\mu$ M resveratrol for 24 h. Luciferase activity was determined by dual luciferase assay (Promega) according to the manufacturer's instructions and normalized to *Renilla* values. The level of survivin promoter activity of untreated cells was set as 100%.

## RESULTS

**Sensitization for TRAIL-Induced Apoptosis by Resveratrol.** In search of novel strategies to target tumor cell resistance, we investigated the antitumor effect of the chemopreventive, natural compound resveratrol on human tumor cell lines. To test the antitumor activity of resveratrol, we selected SHEP neuroblastoma cells, which have previously been proven suitable for studies on apoptosis pathways by our group and by other investigators (23, 33). Treatment of SHEP neuroblastoma cells with resveratrol strongly inhibited proliferation in a dose- and time-dependent manner, whereas resveratrol only moderately induced apoptosis at equimolar concentrations (data not shown). Given the relatively low cytotoxic activity of resveratrol as a single agent, we then tested resveratrol in combination treatments. Surprisingly, we found that resveratrol strongly cooperated with the death ligand TRAIL to induce apoptosis in SHEP neuroblastoma cells in a dose- and time-dependent manner (Fig. 1, A and B). Also, resveratrol cooperated with TRAIL to reduce clonogenic tumor cell growth (Fig. 1C). Because combinations of cytotoxic agents may give rise to different effects, depending on treatment schedules of agents, we tested whether the treatment schedules of resveratrol and TRAIL might influence the cooperative effect. Sequential treatment with resveratrol for 12 h followed by TRAIL for 12 h or with TRAIL for 12 h followed by resveratrol for 12 h yielded less apoptosis than cotreatment with resveratrol and TRAIL for 24 h (data not shown). To exclude the possibility that the sensitization effect of resveratrol was restricted to TRAIL, we tested resveratrol in various combination treatments. Also, resveratrol sensitized SHEP neuroblastoma cells for CD95- or drug-induced apoptosis (Fig. 1D). We then extended our studies to a panel of human cancer cell lines. Importantly, resveratrol sensitized a variety of human cancer cell lines including neuroblastoma, medulloblastoma, glioblastoma, melanoma, breast carcinoma, prostate carcinoma, or pancreatic carcinoma cells to TRAIL-induced apoptosis (Table 1). In contrast, resveratrol failed to sensitize untransformed human fibroblasts to TRAIL-induced apoptosis, indicating some tumor specificity (Table 1). Together, this set of experiments indicate that resveratrol sensitized a variety of different tumor cells to apoptosis on death receptor ligation or treatment with anticancer drugs.

To see whether sensitization by resveratrol occurred in a caspase-dependent manner, we used the broad range caspase inhibitor zVAD-fmk. Resveratrol-mediated sensitization for TRAIL was markedly blocked in the presence of zVAD-fmk, which inhibited caspase-3 activation on treatment with resveratrol and TRAIL, indicating that sensitization by resveratrol was mediated by activation of caspases

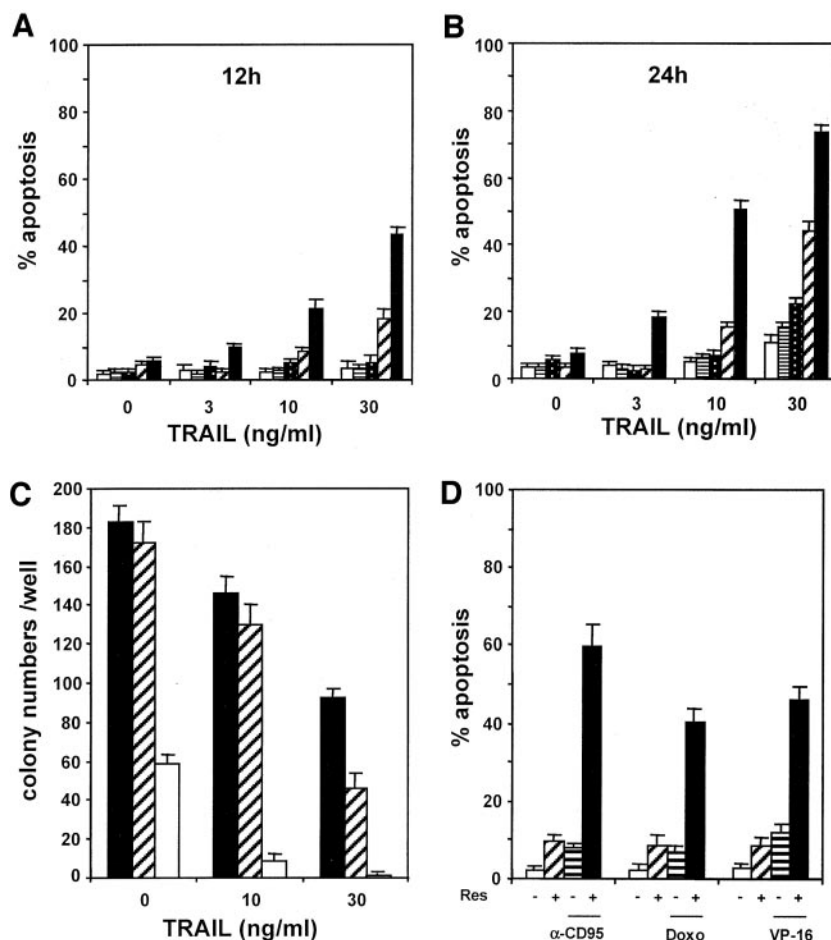


Fig. 1. Sensitization for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by resveratrol. *A* and *B*, effect of resveratrol on TRAIL-induced apoptosis. SHEP neuroblastoma cells were treated with 0–100  $\mu$ M resveratrol (0  $\mu$ M,  $\square$ ; 3  $\mu$ M,  $\blacksquare$ ; 10  $\mu$ M,  $\square$ ; 30  $\mu$ M,  $\blacksquare$ ; 100  $\mu$ M,  $\blacksquare$ ) and/or 0–30 ng/ml TRAIL for 12 h (*A*) or 24 h (*B*). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. *C*, effect of resveratrol on TRAIL-induced clonogenic survival. SHEP neuroblastoma cells were treated with resveratrol (0  $\mu$ M,  $\blacksquare$ ; 10  $\mu$ M,  $\blacksquare$ ; 30  $\mu$ M,  $\square$ ) and/or 0–30 ng/ml TRAIL. Clonogenic survival was determined by crystal violet staining, and colony numbers/well of a 24-well plate are indicated. *D*, effect of resveratrol on death receptor- or drug-induced apoptosis. SHEP neuroblastoma cells were treated with 100  $\mu$ M resveratrol for 24 h, followed by 1  $\mu$ g/ml anti-CD95 antibody, 0.1  $\mu$ g/ml doxorubicin, or 3  $\mu$ g/ml VP16 for 24 h. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei.

(Fig. 2, *A* and *B*). Western blot analysis revealed that resveratrol and TRAIL cooperated in activation of caspase-8, -9, -3, and -7 and cleavage of Bid and DFF45 (Fig. 2*C*, *Lanes 7, 10, and 13*).

**Sensitization for TRAIL-Induced Apoptosis through Resveratrol-Mediated Cell Cycle Arrest.** To gain insight into the molecular mechanism(s) regulating apoptosis sensitivity on treatment with resveratrol, we analyzed in more detail the effect of resveratrol on the cell cycle, given the strong growth-inhibitory effect of resveratrol. Cell cycle analysis revealed accumulation of cells in  $G_1$  phase of the cell cycle on treatment with resveratrol (Fig. 3*A*). To test whether the effects of resveratrol on cell cycle were reversible, cells were incubated with resveratrol for 24 h and thereafter recultured in medium without resvera-

rol. Importantly, removal of resveratrol from the culture medium, which reversed the resveratrol-mediated cell cycle arrest, also reversed the sensitizing effect of resveratrol for TRAIL-induced apoptosis (data not shown). These findings indicate a link between cell cycle regulation and apoptosis sensitivity on treatment with resveratrol.

Therefore, we next examined whether resveratrol-mediated cell cycle arrest was a prerequisite for induction of apoptosis. To this end, cells were double-stained with propidium iodide for cell cycle distribution and annexin V for apoptosis and analyzed by flow cytometry. Treatment with resveratrol alone resulted in  $G_1$  arrest, whereas combined treatment with resveratrol and TRAIL preferentially induced apoptosis in  $G_1$ -arrested cells (Fig. 3*B*).

To directly test the sensitivity at different phases of the cell cycle, cells were arrested in  $G_1$ , S, or  $G_2$  phase by the specific cell cycle inhibitors mimosine, thymidine, or nocodazole (data not shown) and then treated with TRAIL. Arresting cells in  $G_1$  or S phase by pretreatment with nontoxic concentrations of mimosine or thymidine, respectively, sensitized cells for subsequent treatment with TRAIL (Fig. 3*C*). No sensitization for TRAIL was found when cells were arrested in  $G_2$  by pretreatment with nocodazole (Fig. 3*C*). These findings indicate a differential sensitivity for TRAIL-induced apoptosis at different phases of the cell cycle with increased sensitivity in  $G_1$  or S phase consistent with previous findings (34).

#### Modulation of Apoptosis Regulatory Molecules by Resveratrol.

To gain insight into the molecular mechanism(s) linking cell cycle regulation and apoptosis sensitivity on treatment with resveratrol, we investigated cell cycle-regulatory proteins and key molecules of the death receptor or mitochondrial pathway. Treatment with resveratrol

Table 1 Cooperative effect of resveratrol and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in human tumor cell lines

Neuroblastoma (GIMEN, LAN5), medulloblastoma (PSFK), glioblastoma (U373MG and A172), melanoma (SK-Mel and Colo38), pancreatic carcinoma (MiaPaCa2), prostate carcinoma (LNCaP), and breast carcinoma cells (MCF7) or human fibroblasts were left untreated (no Tx) or treated for 24 h with 100  $\mu$ M resveratrol (Res) and/or 30 ng/ml TRAIL. Apoptosis was determined by FACS analysis of propidium iodide-stained DNA.

|                   | no Tx         | Res           | TRAIL         | Res + TRAIL    |
|-------------------|---------------|---------------|---------------|----------------|
| GIMEN             | 3.7 $\pm$ 1.8 | 7.3 $\pm$ 1.4 | 8.7 $\pm$ 1.7 | 45.7 $\pm$ 3.0 |
| LAN5              | 2.1 $\pm$ 0.8 | 2.6 $\pm$ 1.6 | 5.9 $\pm$ 0.9 | 57.2 $\pm$ 2.8 |
| PSFK              | 2.6 $\pm$ 1.6 | 6.4 $\pm$ 1.4 | 6.8 $\pm$ 1.4 | 42.1 $\pm$ 2.9 |
| U373MG            | 2.5 $\pm$ 1.2 | 7.5 $\pm$ 0.4 | 7.0 $\pm$ 1.5 | 39.3 $\pm$ 2.5 |
| A172              | 2.7 $\pm$ 0.7 | 6.7 $\pm$ 2.6 | 3.1 $\pm$ 1.0 | 40.9 $\pm$ 3.1 |
| SK-Mel            | 3.6 $\pm$ 0.6 | 8.8 $\pm$ 1.1 | 7.4 $\pm$ 2.2 | 42.2 $\pm$ 2.6 |
| Colo38            | 4.6 $\pm$ 0.7 | 5.5 $\pm$ 1.1 | 4.4 $\pm$ 1.6 | 47.6 $\pm$ 2.0 |
| MiaPaCa2          | 4.1 $\pm$ 2.4 | 6.6 $\pm$ 2.2 | 5.3 $\pm$ 1.3 | 34.2 $\pm$ 2.2 |
| LNCaP             | 1.3 $\pm$ 0.3 | 5.7 $\pm$ 1.9 | 4.0 $\pm$ 1.1 | 40.9 $\pm$ 3.4 |
| MCF7              | 1.1 $\pm$ 0.9 | 6.1 $\pm$ 1.9 | 5.6 $\pm$ 2.0 | 38.7 $\pm$ 2.5 |
| Human fibroblasts | 1.7 $\pm$ 0.9 | 2.6 $\pm$ 1.3 | 2.5 $\pm$ 1.2 | 3.6 $\pm$ 1.4  |

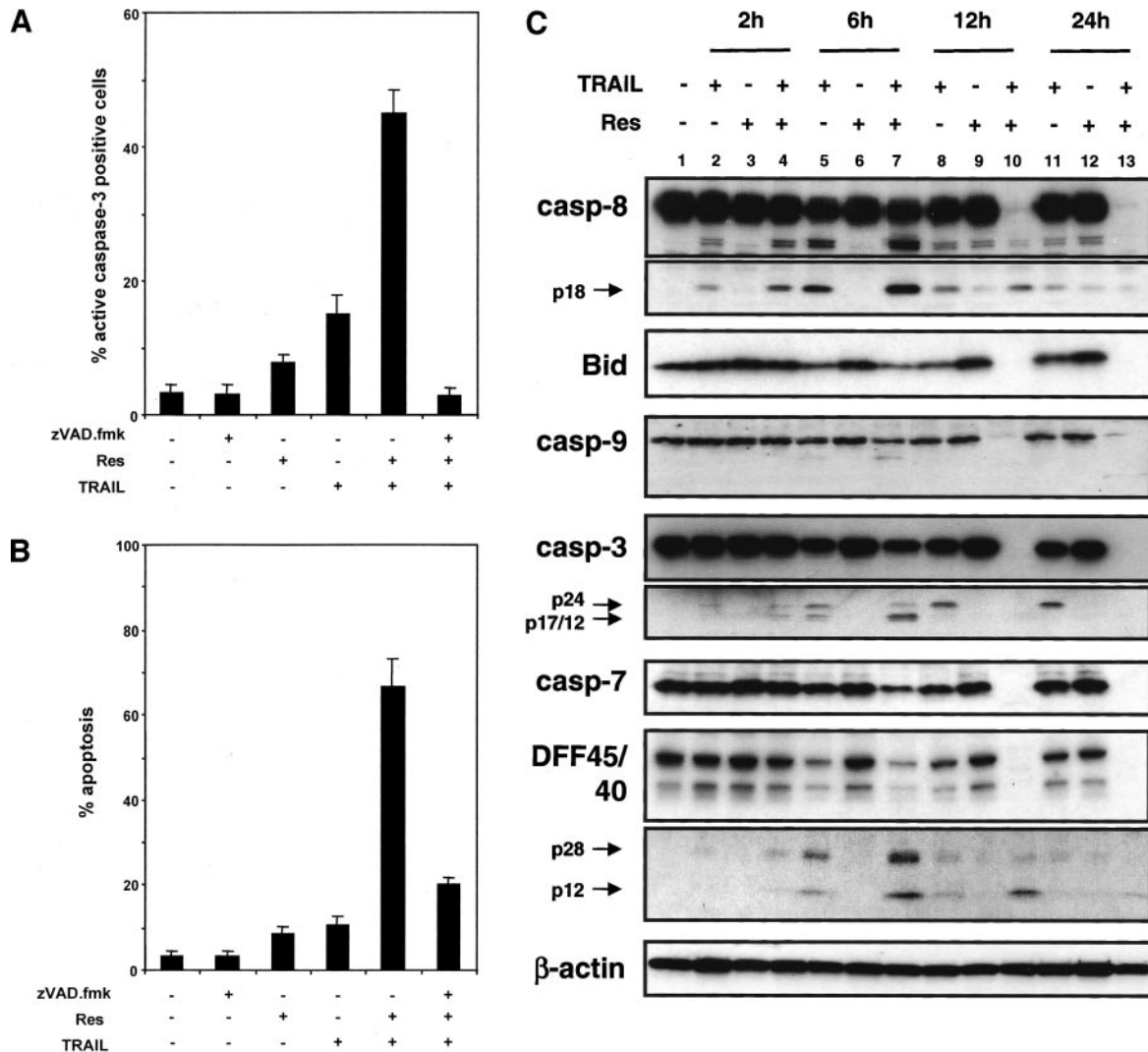


Fig. 2. Resveratrol-mediated sensitization for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis requires caspase activity. *A* and *B*, effect of caspase inhibition on resveratrol-mediated sensitization for TRAIL-induced apoptosis. SHEP neuroblastoma cells were left untreated or treated for 6 h (*A*) or 24 h (*B*) with 100  $\mu$ M resveratrol and/or 30 ng/ml TRAIL in the presence or absence of 50  $\mu$ M zVAD-fmk. Caspase activity was determined by staining active caspase-3 and flow cytometry (*A*). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei (*B*). *C*, effect of resveratrol on TRAIL-induced caspase activation. SHEP neuroblastoma cells were treated with 100  $\mu$ M resveratrol and/or 30 ng/ml TRAIL for the indicated times. Expression of caspase-8, caspase-9, caspase-3, caspase-7, Bid, DFF45/DFF40, and  $\beta$ -actin was assessed by Western blot analysis. Cleavage products are indicated by arrows.

resulted in an increase in p53 and p21 protein expression and rapid down-regulation of survivin protein expression (Fig. 4). Also, treatment with resveratrol led to down-regulation of cyclin D1 protein expression, further supporting the notion that resveratrol induced G<sub>1</sub> arrest (Fig. 4). There was a slight increase in Bax or Bak protein expression on treatment with resveratrol, whereas levels of Bcl-2 or Bcl-X<sub>L</sub> proteins remained unaltered (Fig. 4). Because resveratrol-induced up-regulation of Bax or Bak expression occurred relatively late compared with the onset of apoptosis on combined treatment with resveratrol and TRAIL, up-regulation of Bax or Bak expression may contribute to the cooperative effect of resveratrol and TRAIL but cannot fully explain it. No up-regulation of TRAIL or CD95 ligand or surface expression of agonistic TRAIL receptors was found (Fig. 4; data not shown).

**Sensitization for TRAIL-Induced Apoptosis by Resveratrol Does Not Require Wild-Type p53.** p53 has been implicated in regulation of both cell cycle and apoptosis (6). Because resveratrol treatment resulted in induction of p53 protein (Fig. 4), we asked whether p53 may provide a molecular link between resveratrol-induced cell cycle alterations and apoptosis. To address this question,

we inhibited p53 function using the p53 inhibitor pifithrin- $\alpha$  in SHEP neuroblastoma cells, which harbor wild-type p53. Interestingly, preincubation of SHEP neuroblastoma cells with pifithrin- $\alpha$ , which reduced resveratrol-induced p53 expression, did not interfere with resveratrol-mediated sensitization for TRAIL, whereas pifithrin- $\alpha$  attenuated cisplatin-induced apoptosis (Fig. 5, *A* and *B*). Next, we used wild-type p53 and p53-deficient HCT116 colon carcinoma cells. Importantly, resveratrol similarly sensitized both wild-type p53 and p53-deficient HCT116 colon carcinoma cells for TRAIL-induced apoptosis (Fig. 5C). Also, induction of p21, down-regulation of survivin expression, and cell cycle arrest in G<sub>1</sub> phase in response to treatment with resveratrol was found in both p53<sup>+/+</sup> and p53<sup>-/-</sup> HCT116 colon carcinoma cells, whereas p53 protein expression was only up-regulated in wild-type p53 cells (Fig. 5, *D* and *E*). Likewise, resveratrol sensitized p53-null Saos osteosarcoma cells to TRAIL-induced apoptosis (Fig. 5F). Accordingly, resveratrol triggered p21 up-regulation, cell cycle arrest, and slight down-regulation of survivin expression in Saos cells (Fig. 5, *G* and *H*). Together, this set of data indicates that wild-type p53 function was dispensable for p21 induction, cell cycle arrest, down-regulation of

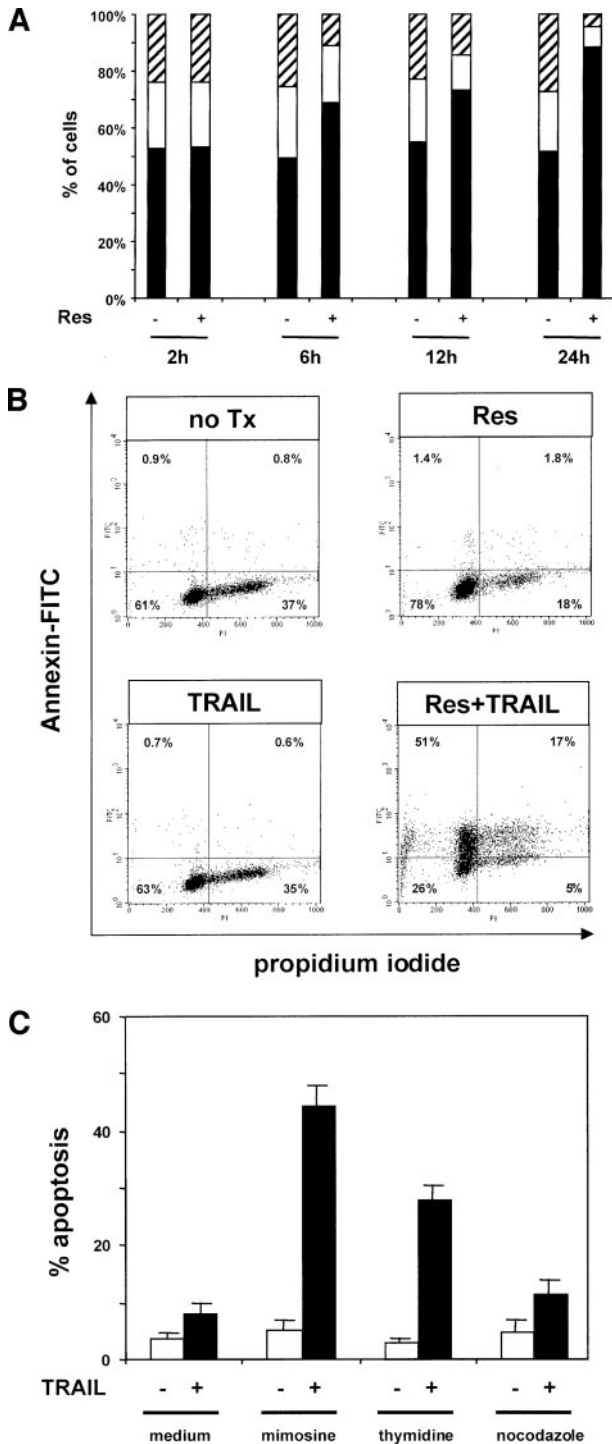


Fig. 3. Effect of resveratrol on cell cycle. **A**, effect of resveratrol on cell cycle distribution. SHEP neuroblastoma cells were left untreated or treated with 100  $\mu$ M resveratrol for 2–24 h. Cell cycle distribution (G<sub>1</sub> phase, ■, S-phase, □, G<sub>2</sub>-M phase, ▨) was assessed as described in “Materials and Methods.” **B**, effect of resveratrol on cell cycle and apoptosis. SHEP neuroblastoma cells were left untreated (*no Tx*) or treated with 100  $\mu$ M resveratrol (*Res*), 30 ng/ml tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*), or 100  $\mu$ M resveratrol and 30 ng/ml tumor necrosis factor-related apoptosis-inducing ligand (*Res+TRAIL*). Concomitant analysis of cell cycle and apoptosis was performed by staining with propidium iodide for cell cycle distribution (DNA content, X axis) and annexin-FITC for apoptosis (Y axis) as described in “Materials and Methods.” The percentage of cells in each quadrant are indicated (*bottom left*, G<sub>1</sub> phase-annexin low; *bottom right*, S/G<sub>2</sub>-M phase-annexin low; *top left*, G<sub>1</sub> phase-annexin high; *top right*, S/G<sub>2</sub>-M phase-annexin high). **C**, effect of cell cycle inhibitors on tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. SHEP neuroblastoma cells were cultured in medium or pretreated for 24 h with 0.4 mM mimosine, 2 mM thymidine, or 0.4  $\mu$ g/ml nocodazole and further cultured in the presence (■) or absence (□) of 30 ng/ml tumor necrosis factor-related apoptosis-inducing ligand for 24 h. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei.

survivin expression, and sensitization for TRAIL-induced apoptosis by resveratrol.

**Sensitization for TRAIL-Induced Apoptosis by Resveratrol through p21-Mediated Cell Cycle Arrest.** Next, we further analyzed the involvement of p21 in the sensitization effect of resveratrol. No detectable up-regulation of *p21* mRNA expression was seen on treatment with resveratrol, despite strong up-regulation of p21 protein expression (Fig. 6A, compare with Fig. 4), indicating that up-regulation of p21 protein by resveratrol occurred primarily through a posttranscriptional mechanism. Interestingly, transfection-enforced overexpression of p21, resulting in cell cycle arrest in G<sub>1</sub> and down-regulation of survivin expression (data not shown), also sensitized SHEP neuroblastoma cells for treatment with TRAIL (Fig. 6B). To further investigate the role of p21 in the cooperative effect of resveratrol and TRAIL, we used *p21* wild-type and *p21*-deficient HCT116 colon carcinoma cells. Importantly, potentiation of TRAIL-induced apoptosis by resveratrol was markedly reduced in *p21*-deficient HCT116 colon carcinoma cells compared with *p21* wild-type cells (Fig. 6C). Accordingly, cell cycle arrest and down-regulation of survivin expression on treatment with resveratrol was reduced in *p21*-deficient cells compared with *p21* wild-type HCT116 colon carcinoma cells (Fig. 6, D and E). Interestingly, although treatment with resveratrol similarly induced p53 expression in both *p21* wild-type and *p21*-deficient HCT116 colon carcinoma cells, the increase in p53 protein expression was not associated with a similar increase in sensitivity to TRAIL-induced apoptosis in *p21*-deficient cells compared with *p21* wild-type cells (Fig. 6, D and E). This set of data

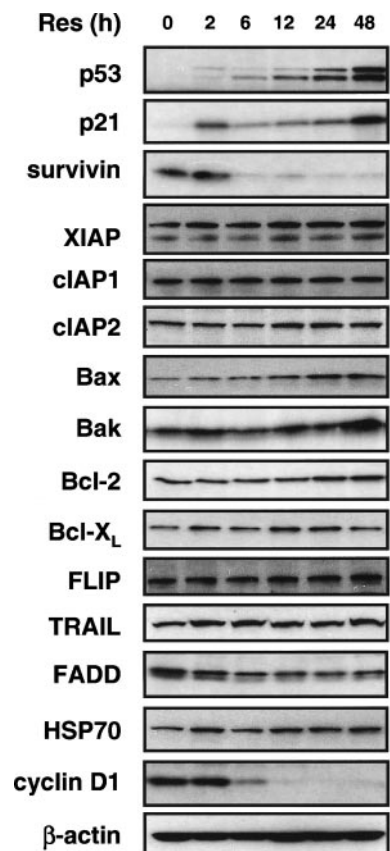


Fig. 4. Effect of resveratrol on apoptosis-regulatory molecules. SHEP neuroblastoma cells were treated with 100  $\mu$ M resveratrol for 0–48 h and assessed for protein expression of p53, p21, survivin, XIAP, cIAP1, cIAP2, Bax, Bak, Bcl-2, Bcl-X<sub>L</sub>, Flip, TRAIL, FADD, HSP70, cyclin D1, and  $\beta$ -actin by Western blot analysis. In addition to XIAP, a nonspecific XIAP immunoreactive molecule migrating slightly larger than XIAP was detected using the mouse monoclonal antibody H62120 (49), which is indicated by the asterisk.

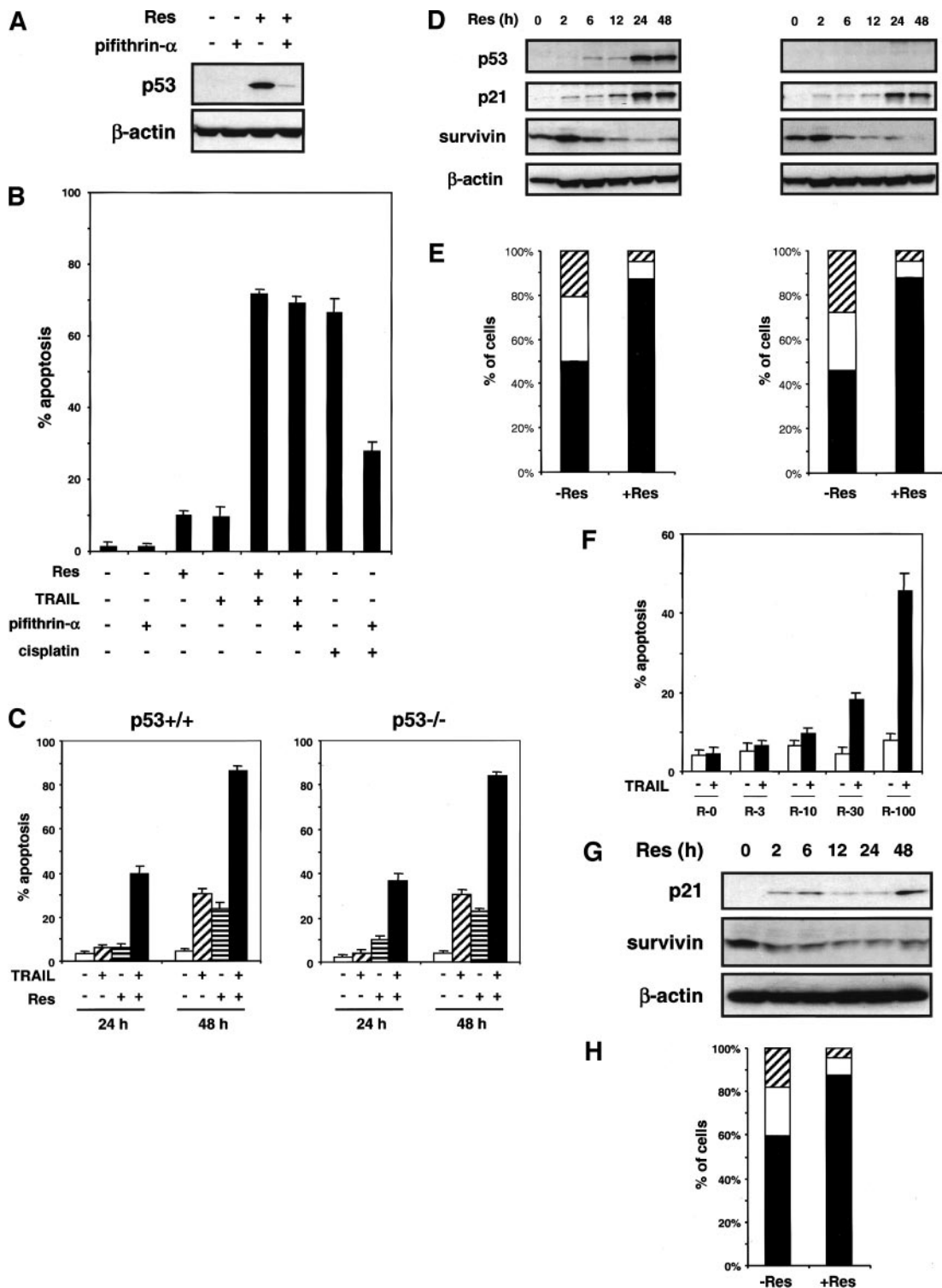


Fig. 5. p53-independent sensitization for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by resveratrol. **A** and **B**, effect of the p53 inhibitor pifithrin- $\alpha$  on resveratrol-mediated sensitization for TRAIL-induced apoptosis. SHEP neuroblastoma cells were left untreated or treated for 24 h with 100  $\mu$ M resveratrol, 30 ng/ml TRAIL, and/or 30  $\mu$ M pifithrin- $\alpha$  or with 10  $\mu$ g/ml cisplatin and/or 30  $\mu$ M pifithrin- $\alpha$  for 72 h. p53 protein expression was determined by Western blot analysis (**A**), and apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei (**B**). **C**, effect of resveratrol on TRAIL-induced apoptosis in p53-deficient colon carcinoma cells. Wild-type p53 (p53<sup>+/+</sup>; left panel) or p53-deficient (p53<sup>-/-</sup>; right panel) HCT116 colon carcinoma cells were treated for 24 or 48 h with 100  $\mu$ M resveratrol and/or 30 ng/ml TRAIL. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. **D**, effect of resveratrol on apoptosis-regulatory molecules in p53-deficient colon carcinoma cells. Wild-type p53 (p53<sup>+/+</sup>; left panel) or p53-deficient (p53<sup>-/-</sup>; right panel) HCT116 colon carcinoma cells were treated with 100  $\mu$ M resveratrol for 0–48 h and assessed for protein expression of p53, p21, survivin, and  $\beta$ -actin by Western blot analysis. **E**, effect of resveratrol on cell cycle in p53-deficient colon carcinoma cells. Wild-type p53 (p53<sup>+/+</sup>; left panel) or p53-deficient (p53<sup>-/-</sup>; right panel) HCT116 colon carcinoma cells were treated with 100  $\mu$ M resveratrol for 24 h. Cell cycle distribution (G<sub>1</sub> phase, ■; S phase, □; G<sub>2</sub>-M phase, ▨) was assessed as described in “Materials and Methods.” **F**, effect of resveratrol on TRAIL-induced apoptosis in p53-null osteosarcoma cells. Saos p53-null osteosarcoma cells were treated for 24 h with 0–100  $\mu$ M resveratrol (R-0 to R-100) in the presence (+) or absence (-) of 30 ng/ml TRAIL. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. **G**, effect of resveratrol on apoptosis-regulatory molecules in p53-null osteosarcoma cells. Saos p53-null osteosarcoma cells were treated with 100  $\mu$ M resveratrol for 0–48 h and assessed for protein expression of p21, survivin, and  $\beta$ -actin by Western blot analysis. **H**, effect of resveratrol on cell cycle in p53-null osteosarcoma cells. Saos p53-null osteosarcoma cells were treated with 100  $\mu$ M resveratrol for 24 h. Cell cycle distribution (G<sub>1</sub> phase, ■; S phase, □; G<sub>2</sub>-M phase, ▨) was assessed as described in “Materials and Methods.”

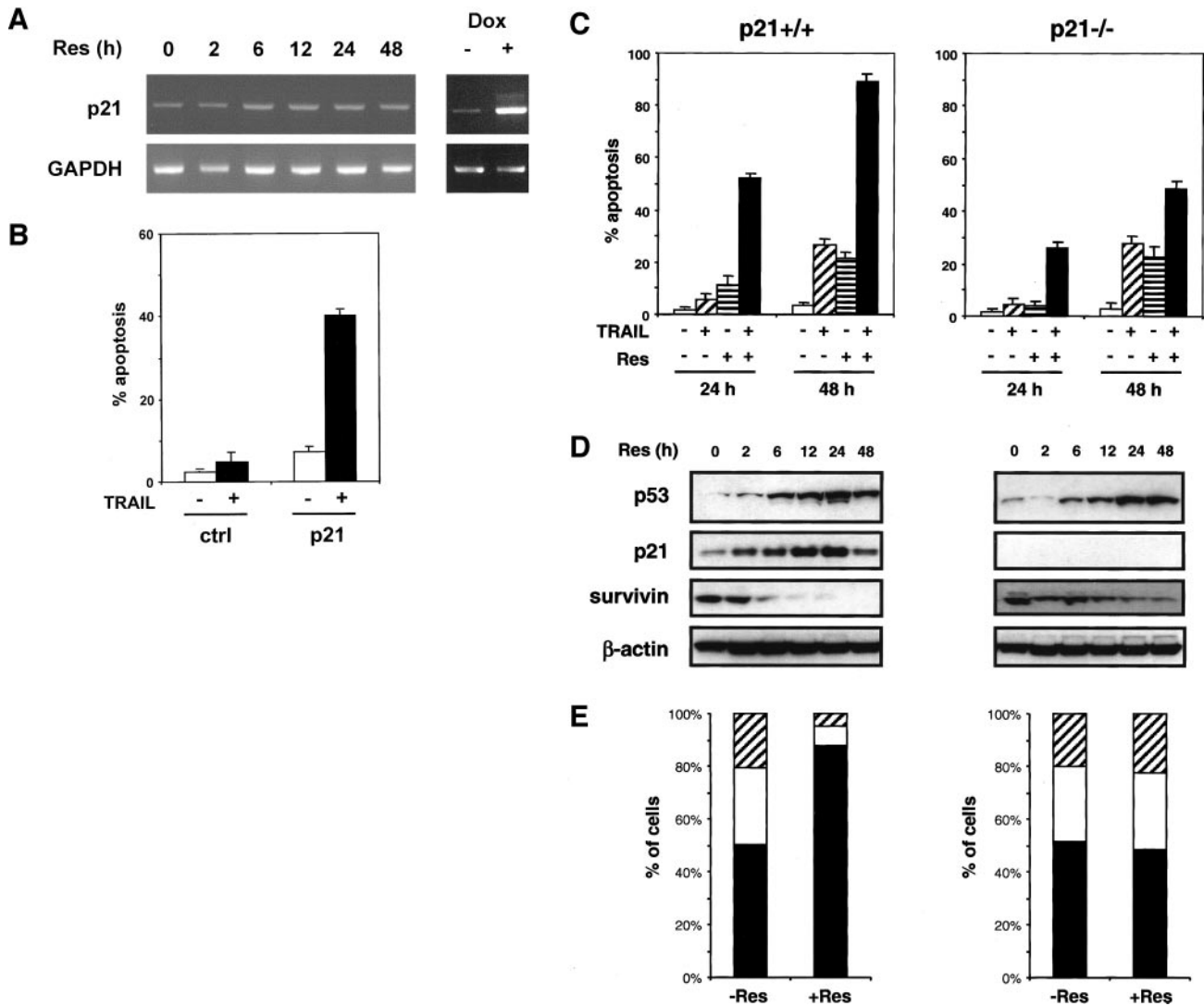


Fig. 6. Sensitization for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by resveratrol involves p21. **A**, effect of resveratrol on p21 mRNA expression. SHEP neuroblastoma cells were treated for 0–48 h with 100  $\mu$ M resveratrol or for 24 h with 0.5  $\mu$ g/ml doxorubicin and assessed for p21 mRNA expression by reverse transcription-PCR. Treatment with doxorubicin was used as positive control for p21 mRNA induction, and glyceraldehyde-3-phosphate dehydrogenase mRNA expression was used to control for equal gel loading. **B**, effect of p21 overexpression on TRAIL-induced apoptosis. SHEP neuroblastoma cells were transiently transfected with p21 cDNA (p21) or empty vector (ctrl) and analyzed after treatment with 30 ng/ml TRAIL for 24 h for apoptosis by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. **C**, effect of resveratrol on TRAIL-induced apoptosis in p21-deficient colon carcinoma cells. Wild-type p21 (p21<sup>+/+</sup>; left panel) or p21-deficient (p21<sup>-/-</sup>; right panel) HCT116 colon carcinoma cells were treated for 24 h or 48 h with 100  $\mu$ M resveratrol and/or 30 ng/ml TRAIL. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. **D**, effect of resveratrol on apoptosis-regulatory molecules in p21-deficient colon carcinoma cells. Wild-type p21 (p21<sup>+/+</sup>; left panel) or p21-deficient (p21<sup>-/-</sup>; right panel) HCT116 colon carcinoma cells were treated with 100  $\mu$ M resveratrol for 0–48 h and assessed for protein expression of p53, p21, survivin, and  $\beta$ -actin by Western blot analysis. **E**, effect of resveratrol on cell cycle in p21-deficient colon carcinoma cells. Wild-type p21 (p21<sup>+/+</sup>; left panel) or p21-deficient (p21<sup>-/-</sup>; right panel) HCT116 colon carcinoma cells were treated with 100  $\mu$ M resveratrol for 24 h. Cell cycle distribution (G<sub>1</sub> phase, ■; S phase, □; G<sub>2</sub>-M phase, ▨) was assessed as described in “Materials and Methods.”

indicates that p21 is involved in cell cycle arrest, survivin depletion, and potentiation of TRAIL-induced apoptosis on treatment with resveratrol independently of p53.

#### Sensitization for TRAIL-Induced Apoptosis by Down-Regulation of Survivin through Resveratrol-Mediated Cell Cycle Arrest.

To gain further insight into the mechanisms linking cell cycle control and apoptosis, we investigated the role of survivin in the sensitization effect of resveratrol. Treatment with resveratrol resulted in slight reduction of survivin mRNA expression (Fig. 7A). Accordingly, treatment with resveratrol led to inhibition of survivin promoter activity compared with untreated cells (Fig. 7B). To test the effect of resveratrol on survivin protein stability, we used cycloheximide block in SHEP neuroblastoma cells transfected with survivin cDNA fused to GFP. Interestingly, expression of the GFP-containing survivin fusion protein was strongly reduced on treatment with resveratrol compared with untreated cells (Fig. 7C). Also, down-regulation of survivin

protein levels by resveratrol was inhibited in the presence of the proteasome inhibitor lactacystin (Fig. 7D). These findings indicate that survivin expression was regulated by resveratrol through transcriptional and posttranscriptional mechanisms. Because we also found down-regulation of survivin expression by arresting cells in G<sub>1</sub> phase by the cell cycle inhibitor mimosine (data not shown) consistent with previous reports on the cell cycle-dependent expression of survivin (21), we asked whether resveratrol would lead directly to survivin depletion or whether decreased survivin expression would be a consequence of the effect of resveratrol on the cell cycle. To address this question, survivin expression and cell cycle status were analyzed simultaneously by flow cytometry. Untreated cells in G<sub>1</sub> exhibited low levels of survivin. Also, in resveratrol-treated cells, decreased expression of survivin was associated with G<sub>1</sub>-arrested cells (Fig. 7E), suggesting that down-regulation of survivin expression by resveratrol occurred as a result

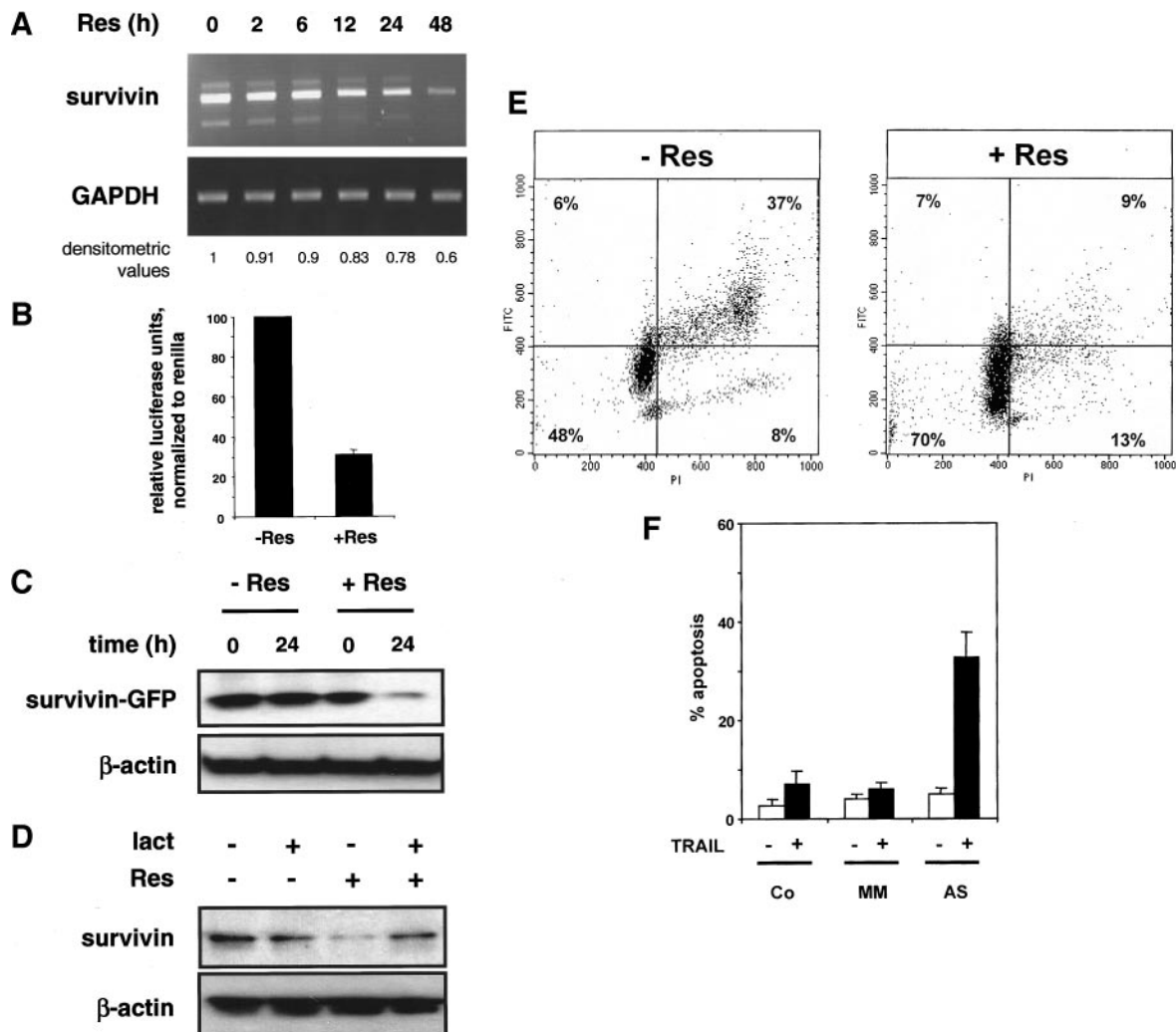


Fig. 7. Sensitization for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by resveratrol involves survivin. **A**, effect of resveratrol on *survivin* mRNA expression. SHEP neuroblastoma cells were treated with 100  $\mu$ M resveratrol for 0–48 h and assessed for *survivin* mRNA expression by reverse transcription-PCR using primers, which detect *survivin* and its isoforms *survivin-2B* and *survivin- $\Delta$ Ex3* (42). *Survivin* expression was quantified by densitometric analysis as described in “Materials and Methods.” Densitometric values are expressed as fold change relative to control. *Glyceraldehyde-3-phosphate dehydrogenase* mRNA expression (*GAPDH*) was used to control for equal gel loading. **B**, effect of resveratrol on *survivin* promoter activity. SHEP neuroblastoma cells were transiently transfected with a vector containing the *survivin* promoter ligated to the promoterless firefly luciferase gene (pGL2-*survivin*) together with *Renilla* luciferase. Sixteen h after transfection, cells were treated (+*Res*) or not treated (–*Res*) with 100  $\mu$ M resveratrol for 24 h. Luciferase activity was determined by dual luciferase assay and normalized to *Renilla* values. The level of *survivin* promoter activity of untreated cells was set as 100%. **C**, effect of resveratrol on *survivin* expression after cycloheximide block. SHEP neuroblastoma cells were transiently transfected with *survivin* fused to GFP. Sixteen h after transfection, cells were treated (+*Res*) or not treated (–*Res*) with 100  $\mu$ M resveratrol in the presence of cycloheximide, harvested at the indicated time intervals and analyzed with antibodies to GFP for *survivin* expression by Western blot analysis.  $\beta$ -Actin protein expression was used to control for equal gel loading. **D**, effect of proteasome inhibitors on *survivin* expression. SHEP neuroblastoma cells were treated for 24 h with 100  $\mu$ M resveratrol and/or 20  $\mu$ M of the proteasome inhibitor lactacystin (*lact*) and analyzed for *survivin* or  $\beta$ -actin expression by Western blot analysis. **E**, effect of resveratrol on cell cycle and *survivin* expression. SHEP neuroblastoma cells were treated with 100  $\mu$ M resveratrol for 24 h. Concomitant analysis of cell cycle and *survivin* expression was performed by staining with propidium iodide (PI; X axis) and FITC-labeled *survivin* (Y axis) as described in “Materials and Methods.” **F**, effect of *survivin* antisense oligonucleotides on TRAIL-induced apoptosis. SHEP neuroblastoma cells were left untreated (Co) or treated with 600 nM *survivin* antisense oligonucleotides (AS) or mismatched oligonucleotides (MM) for 24 h and analyzed after treatment with 30 ng/ml TRAIL for 24 h for apoptosis by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei.

of cell cycle arrest. Because the role of *survivin* in regulating apoptosis sensitivity of tumor cells has been controversially discussed (21), we tested whether or not down-regulation of *survivin* would have an effect on TRAIL-induced apoptosis in SHEP neuroblastoma cells. Importantly, down-regulation of *survivin* expression by *survivin* antisense oligonucleotides (data not shown) sensitized cells to TRAIL-induced apoptosis (Fig. 7F). This indicates that down-regulation of *survivin* by resveratrol-induced  $G_1$  arrest may account, at least in part, for the sensitization effect of resveratrol for TRAIL-induced apoptosis.

## DISCUSSION

Because resistance of many tumors to established treatment regimens still constitutes a major concern in oncology, attempts to im-

prove the survival of cancer patients depend largely on strategies to target tumor cell resistance (1). Induction of apoptosis in cancer cells is a key mechanism for most antitumor therapies including chemotherapy,  $\gamma$ -irradiation, immunotherapy, or cytokines, and thus, defects in apoptosis programs may cause resistance (3–7). *Survivin* is a member of the IAP family that is expressed at high levels in most human cancers including neuroblastoma. *Survivin* may contribute to resistance of tumors by facilitating evasion from apoptosis and aberrant mitotic progression (21). Because inhibition of caspases by IAPs such as *survivin* occurs in the effector phase of apoptosis, a central point where multiple signaling pathways converge, therapeutic modulation of *survivin* could target a key mechanism of cancer resistance (20–22).

Naturally occurring antioxidants such as resveratrol have been



suggested as cancer-preventive agents (24, 25). In the search for strategies to overcome resistance, we tested the antitumor activity of resveratrol on human tumor cell lines. We found that the chemopreventive agent resveratrol is a potent sensitizer of tumor cells for TRAIL-induced apoptosis through p21-mediated cell cycle arrest and associated survivin depletion. This conclusion is supported by several independent pieces of evidence.

#### **Sensitization for TRAIL-Induced Apoptosis by Resveratrol-Induced Cell Cycle Arrest and Concomitant Survivin Depletion.**

Treatment with resveratrol caused p21-mediated G<sub>1</sub> arrest, which in turn resulted in survivin depletion and sensitization for TRAIL-induced apoptosis. Accordingly, transfection-enforced overexpression of p21 induced G<sub>1</sub> arrest, resulting in survivin depletion and sensitization for TRAIL-induced apoptosis. Consequently, cell cycle arrest, survivin depletion, and sensitization to TRAIL-induced apoptosis by resveratrol were all impaired in *p21*-deficient cells. Because survivin expression was almost exclusively down-regulated in G<sub>1</sub>-arrested cells on treatment with resveratrol, our findings suggest that resveratrol indirectly down-regulated survivin levels through its effect on cell cycle. Thus, survivin depletion by resveratrol may reflect cell cycle arrest. Importantly, down-regulation of survivin in G<sub>1</sub> phase resulted in tumor cell death preferentially out of G<sub>1</sub> phase on combined treatment with resveratrol and TRAIL. Likewise, down-regulation of survivin expression by survivin antisense oligonucleotides sensitized cells to TRAIL-induced apoptosis. These findings indicate that cell cycle arrest and associated survivin depletion may account for the sensitization effect of resveratrol for TRAIL treatment. Interestingly, resveratrol-mediated cell cycle arrest and survivin depletion as well as sensitization for TRAIL occurred independently of the *p53* status because these effects were found in *p53*-deficient or *p53*-null cells. Also, up-regulation of p21 protein expression occurred without a detectable increase in *p21* mRNA expression. This indicates that p21 protein expression was regulated by resveratrol through a posttranscriptional mechanism independent of p53. p21 has been reported to promote or to inhibit apoptosis, depending on the cell type and/or stimulus (35, 36). Although resveratrol has previously been shown to induce cell cycle arrest, growth inhibition, or apoptosis in human cancer cell lines, our study considerably extended these earlier studies by showing for the first time that the effect of resveratrol on the cell cycle can be exploited in combination regimens to render tumor cells more susceptible to TRAIL-induced apoptosis.

Several studies have addressed the issue of cell cycle-dependent apoptotic sensitivity of tumors (37, 38). Interestingly, the few studies on cell cycle-dependent regulation of TRAIL sensitivity reported enhanced sensitivity of G<sub>1</sub>-arrested cancer cells for TRAIL (34, 39, 40). In the past, most concepts on cell cycle-dependent action of anticancer drugs were focused on the machinery for DNA synthesis and replication (37, 38). However, the key finding that killing of tumor cells by anticancer agents critically requires activation of apoptosis systems may provide an alternative explanation. Thus, a molecular link between differential drug sensitivity at distinct phases of the cell cycle may be attributed to the differential expression of apoptosis-regulatory proteins in each phase (37, 38). Our findings demonstrate that cell cycle arrest on treatment with resveratrol resulted in down-regulation of survivin expression through transcriptional and posttranscriptional mechanisms by inhibiting promoter activity, decreasing protein stability, and enhancing proteasomal degradation. Although the role of survivin in apoptosis inhibition has been well established in various systems (21), the question of whether survivin functions in cytoprotection or in regulation of cell division has also been controversially discussed, and the answer may vary between different cell types. Our findings showing that down-regulation of survivin expression by antisense oligonucleo-

tides enhanced TRAIL-induced apoptosis similar to resveratrol support the notion that resveratrol sensitized the tumor cell lines used in our studies for apoptosis, at least in part, by cell cycle-dependent depletion of survivin levels. Interestingly, resveratrol treatment did not affect the levels of other IAPs (XIAP and cIAP1/2), emphasizing the specific effect of resveratrol on cell cycle and survivin expression. Because survivin is a nuclear factor  $\kappa$ B target gene, and resveratrol has been shown to down-regulate nuclear factor  $\kappa$ B, inhibition of nuclear factor  $\kappa$ B activity by resveratrol may also contribute to suppression of survivin expression on treatment with resveratrol (41).

**Sensitizer (Resveratrol)/Inducer (TRAIL) Strategy for Experimental Cancer Therapy.** Whereas the ability of resveratrol as a single agent to induce apoptosis was relatively low compared with its profound antiproliferative effect, the combination of resveratrol as "sensitizer" together with the death ligand TRAIL as "inducer" strongly cooperated to trigger apoptosis. The potential of resveratrol for anticancer therapy may therefore largely reside in its ability to sensitize tumor cells for cell death. The sensitizing effect of resveratrol for apoptosis was not restricted to TRAIL but was also found in response to CD95 ligation or anticancer drug treatment. Thus, this combined sensitizer (resveratrol)/inducer (*e.g.*, TRAIL) strategy may be a novel approach to render tumor cells more susceptible to death induction.

Our findings may have several important implications, *e.g.*, for the proposed use of TRAIL in cancer therapy. Recombinant soluble TRAIL is a promising candidate for cancer therapy because it induces apoptosis in a broad spectrum of cancer cell lines, but not in normal cells (42–47). Because resveratrol significantly potentiated the cytotoxic activity of TRAIL even at relatively low TRAIL concentrations, resveratrol may be used in TRAIL-based therapies to reduce the doses of TRAIL required for inhibition of tumor growth. Also, resveratrol may be particularly useful to sensitize resistant tumors to TRAIL-induced apoptosis. Of note, resveratrol did not reverse the lack of toxicity of TRAIL on primary nonmalignant human cells, *e.g.*, fibroblasts. The underlying mechanisms for the differential sensitivity of malignant *versus* nonmalignant human cells toward the sensitization effect by resveratrol are presently unknown and are subject to further investigation.

In a more general application, the dietary compound resveratrol may serve as a novel therapeutic to target survivin expression in cancers through p21-mediated cell cycle arrest. Importantly, we found that resveratrol sensitized a variety of human cancer cell lines to TRAIL-induced apoptosis independent of wild-type *p53* status through cell cycle arrest-mediated survivin depletion.

The potential clinical implications of our studies will also depend on whether or not resveratrol can be given safely to humans at doses high enough to achieve pharmacologically active levels. Toxicity data for resveratrol, although limited at present, showed minimal toxicity to human peripheral blood cells and no overt toxicities in experimental animal studies (48). Clinically, resistance to apoptosis is a major cause of nonresponsiveness of cancers, leading to treatment failure. Thus, in terms of a clinical perspective, the combination of resveratrol and TRAIL may be a novel strategy for the treatment of a variety of human cancers that warrants further investigation.

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