Full Paper

Molecular mechanisms of resveratrol-induced apoptosis in human pancreatic cancer cells

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Abstract: Resveratrol is a polyphenolic phytoalexin found at high concentrations in grapes, nuts, fruits and red wine with reported anti-carcinogenic effects. In this study, the molecular mechanism of resveratrol-induced apoptosis in human pancreatic cancer (Panc 2.03) cells is investigated. Resveratrol treatment of Panc 2.03 cells results in dose-dependent inhibition of cell growth and cells accumulated at the S phase transition of the cell cycle. The anti-proliferative effect of resveratrol is due to apoptosis as seen by the appearance of chromatin condensation, nuclear fragmentation, DNA ladder formation and increased annexin V-stained cells. The apoptotic process is induced by decreased *Bcl-2* expression concomitant with increased *Bax* expression, leading to an increase in the *Bax/Bcl-2* ratio and subsequent activation of caspase-9 and caspase-3. In addition, resveratrol treatment also decreases the *survivin* level and increases the apoptosis-inducing factor level in a dose-dependent manner. These results suggest that resveratrol induces apoptosis of Panc 2.03 cells, at least in part through a mitochondrial-associated intrinsic pathway in both caspase-dependent and independent manners. The present findings suggest that resveratrol has potential as a chemopreventive agent, and possibly as a therapeutic one against pancreatic cancer.

Keywords: pancreatic cancer (Panc 2.03) cell, resveratrol, apoptosis, S phase arrest, *Bcl-2*, *Bax*, *survivin,* apoptosis-inducing factor

INTRODUCTION

Pancreatic cancer is the eighth most common cause of cancer-related deaths worldwide [1, 2]. It has an extremely poor prognosis; the respective 1- and 5-year relative survival rates are 25% and 6%. Treatments for pancreatic cancer remain limited, as only localised cancer is considered suitable for surgery, radiotherapy or chemotherapy [3, 4]. Alternative modes of therapy are therefore needed. Efforts have been made to develop novel strategies including chemoprevention using dietary phytochemicals [4]. Resveratrol (*trans*–3, 4$'$, 5–trihydroxystilbene) is a natural polyphenolic phytoalexin present in grapes, nuts, berries, red wine and various plants [5, 6] with a variety of pharmacological properties, i.e. cardioprotective, neuroprotective, anti-oxidant, antiviral, anti-inflammatory, anti-mutagenic, anti-carcinogenic and anticancer activities [7-10].

The cell processes of growth arrest, apoptosis, proliferation or differentiation are highly regulated by numerous proteins and signalling pathways. Disturbance of these processes can avert cell death and lead to cancer [11]. Moreover, disruption of the apoptotic pathway can confer resistance among cancer cells to radiation and chemotherapy [12]. Pancreatic cancer is a multi-stage process caused by the accumulation of genetic changes of normal cells leading to disturbance in cell cycle regulation and continuous growth [13]. Others have reported resveratrol-induced cell cycle arrest and apoptosis through (a) up-regulation of p53 and p21, (b) down-regulation of Bcl-2 expression and (c) activation of caspase-3 in several pancreatic cancer cell lines [10, 14]. Since there has been no study of the growth inhibition by resveratrol of Panc 2.03 pancreatic cancer cells, we investigate the inhibition of proliferation and apoptotic induction by resveratrol in Panc 2.03 cells. The molecular mechanisms of resveratrol-induced proliferation arrest and apoptosis are also explored.

MATERIALS AND METHODS

Cell Culture

The human pancreatic adenocarcinoma cell line, Panc 2.03, was provided by Dr. E. M. Jaffee of the Sol Goldman Pancreatic Cancer Research Centre, Johns Hopkins University, Baltimore, MD, USA. Cells were cultured in RPMI 1640 (Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin and 100 units/mL penicillin (Gibco BRL) in an incubator at 37°C with a humidified atmosphere containing 5% CO_2.

Cell Growth Inhibition Assay

The growth inhibitory effect of resveratrol was determined using the sulphorhodamine B assay. The Panc 2.03 cells (2×10^3 cells/well) were seeded in 96-well microtitre plates and incubated for 24 hr (day 0). The cells were treated with 2.5, 5, 10 and 20 μg/mL of resveratrol (Sigma-Aldrich, USA) in cell culture medium, and with 0.1% DMSO as the solvent control. The plates were incubated at 37°C for 48 hr (day 2). Cells were fixed with trichloroacetic acid and stained with 0.4% sulphorhodamine B. The bound dye was solubilised with Tris-base buffer. The absorbance (OD) was measured using an ELISA plate reader (Sunrise-TECAN, USA) at 510 nm. The percentage of cell viability was calculated using equation: % cell survival = [OD $_{\text{test sample day 2}}$− OD $_{\text{day 0}}$] x 100 /[OD $_{\text{control day 2}}$− OD $_{\text{day 0}}$]. The 50% inhibitory concentration (IC_{50}) was then determined.

Cell Cycle Distribution Assay

Cells (1×10^6) were plated in duplicate in 10-cm dishes (Corning, USA), then incubated at 37°C for 24 hr. They were cultured in complete medium or 0.1% DMSO or 10, 20, 40 and 80 µg/mL resveratrol for 48 hr. After treatment, the cells were washed with cold phosphate buffer saline, fixed in 70% ethanol at 4°C overnight, and then stained with 200 µL GUAVA® cell cycle reagent (GUAVA Technologies, USA) at room temperature (~26°C) for 30 min. in the dark according to the manufacturer's instructions. The cell cycle distribution (using 10,000 cells per analysis) was analysed in 3 different experiments by a FACSCalibur flow cytometer (Becton Dickinson Bioscience, USA). The percentage distribution of cell cycle phases was analysed by CellQuest software (Becton Dickinson, NJ, USA).

Morphological Examination

Cells (1×10^6) were grown in a 25-mL flask at 37°C for 24 hr, then starved in medium containing 0.5% fetal bovine serum for another 24 hr prior to treatment with 0.1% DMSO or 40 µg/mL resveratrol for 48 hr. Cell morphological changes were observed under a bright-field inverted Nikon microscope. In preparation for the nuclear morphology study, the treated cells were washed with phosphate buffer saline, fixed with methanol, stained with 1 µg/mL 4,6-diamidino-2-phenylindole solution (Roche, Germany) at room temperature for 10 min. and observed under a Nikon fluorescent microscope.

The percentage of apoptotic cells was determined using an annexin V-FITC apoptosis detection kit (BD Pharmingen^{TM}, USA) [15]. The cells (5×10^5 cells/well) were seeded in a 6-well culture plate (Corning, USA) for 24 hr, starved for 24 hr and treated with 0.1% DMSO or 20, 40 and 80 µg/mL resveratrol. After treatment, both adherent and floating cells were harvested and then double-labelled with annexin V-FITC and propidium iodide for 15 min. Ten thousand events were analysed for each sample using flow cytometry on a FACSCalibur and CellQuest software (Becton Dickinson, NJ, USA) [15].

DNA Fragmentation Assay

DNA fragmentation was determined according to Herrmann et al. [16] with some modifications. After the cells (1×10^6) were cultured for 24 hr and starved in medium containing 0.5% fetal bovine serum for 24 hr, they were treated with 0.1% DMSO or 20, 40 and 80 µg/mL resveratrol or 10 µg/mL etoposide as a positive control for 48 hr. The fragmented DNA in cell lysate was purified using a QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA fragments were precipitated with ethanol, re-suspended in 50 µL of Tris EDTA buffer and analysed by 1.6% agarose gel electrophoresis.

Quantitative Real-time Reverse-transcription Polymerase Chain Reaction

Cells were treated with 0.1% DMSO or 40 µg/mL resveratrol for 12 and 24 hr. Total cellular RNA was extracted using TRIzol reagent (Invitrogen, USA) as per the manufacturer's instructions. The reverse-transcription reaction was carried out [17], and then real-time polymerase chain reaction (PCR) was performed for the indicated genes (*Bcl-2*, *Bax*, *survivin* and *GAPDH*) [17] in a 20-µL PCR mixture which contained first-strand cDNA, 5 *p*moles of each primer and 10 µL of 2×SYBR Green PCR Master Mix (Gene System Co., USA), employing an ABI 7500 Real-time PCR System (Applied Biosystems, USA).

The sequences for the specific primers used in the PCR are summarised in Table 1. The sequences of PCR products were obtained by direct sequencing. Each amplicon was then cloned into pGEM®-T vector (Promega, USA) in order to generate standard curves for target cDNA. The respective ratio of copy numbers of each target cDNA to *GAPDH* cDNA was reported as the mRNA level. The up- or down-regulation of gene expression was determined according to the ratio of fold-change in gene expression between the treated and control cells.

Table 1. Primer sequences used in real-time PCR analysis

Gene	Forward primer	Reverse primer
Bcl-2	5′TGGATGACTGAGTACCTGA3′	5′TGAGCAGAGTCTTCAGAGA3′
Survivin	5′AAGGCTGGGAGCCAGA3′	5′TGGCTCTTTCTCTGTCCA3′
Bax	5′AACCATCATGGGCTGGA3′	5′CGCCACAAAGATGGTCA3′
GAPDH	5′TCATCAGCAATGCCTCCTGCA3′	5′TGGGTGGCAGTGATGGCA3′

Protein Extraction and Western Blot Analysis

Cells (2×10^6) were treated with 0.1% DMSO or 10, 20, 40 and 80 µg/mL resveratrol for 48 hr, and then harvested and lysed in ice-cold RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, 0.1% sodium dodecyl sulphate, 1 mM E-64, 2 mM phenylmethylsulphonyl fluoride and 2 mM leupeptin. After homogenisation and centrifugation at 13,000 *g,* 4ºC, for 30 min., the supernatant was collected as whole cell lysate and the protein concentration was determined using a Coomassie protein assay kit (Pierce Biotechnology Inc., USA). Equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, UK). After blocking, the membranes were incubated overnight with the primary antibodies against Bcl-2, Bax, survivin, apoptosis inducing factor (Santa Cruz Biotechnology, USA) or procaspase-9 and procaspase-3, activated caspase-9 (Cell Signaling, USA) or activated caspase-3, and *β*-actin (Sigma USA) at 4ºC. The membranes were then incubated at room temperature for 1 hr with the corresponding secondary antibodies, viz. horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology, USA). The blots were detected with an enhanced chemiluminescence kit (Pierce Biotechnology, USA) and fluorogrammed with CL-XPosure film. The protein band intensity was quantified using Scion Image software. For every immunoblot, equal loading of protein was confirmed by stripping the blot and re-probing with *β*-actin antibody. The relative intensity was evaluated and normalised with *β*-actin.

Statistical Analysis

Values are presented as mean ± SE. The *Student's t*-test was performed to determine the significance between untreated control cells and the resveratrol-treated cells. Differences were considered significant at *p* < 0.05. All analyses were performed using SPSS version 10.0 (SPSS Inc., USA).

RESULTS AND DISCUSSION

Growth Inhibitory Effect of Resveratrol on Panc 2.03 Cells

We first determine the effect of resveratrol on the growth of Panc 2.03 cells *in vitro*. The respective cell survival rates among the Panc 2.03 cells treated with resveratrol at 2.5, 5, 10 and 20 μg/mL (11-87.6 μM) are 54, 50, 43 and 30% respectively (Figure 1). Similar to observations of a variety of pancreatic cancer cell lines, our study shows that after the 48-hr exposure to 2.5-20 μg/mL (11-87.6 μM) of resveratrol, Panc 2.03 cells exhibit marked growth inhibition in a dose-dependent manner (Figure 1). A similar dose range of 25-200 μM has been observed to inhibit the proliferation of various types of pancreatic cancer cell lines [18-20]. Panc 2.03 cells are relatively sensitive to the cytotoxic effects of resveratrol as suggested by the IC_{50} values of only 5 μg/mL (22 μM) against 51-100 μM reported for other types of pancreatic cancer cells (Panc-1, CFPAC-1, MIA Paca-2, AsPC-1, BxPC-3, S2-013 and CD 18 cells) [18-20].

The difference in the sensitivity of pancreatic cancer cell lines to mitomycin C partly depends on which gene(s) controls the Fanconi Anemia (FA) pathway [21]. Specifically, mutations in the FA genes *FANCC*, *FANCG* and *BRCA2* have been reported in pancreatic cancer cell lines [21, 22] and the FA-defective pancreatic cancer cell lines—Hs 766T (*FANCG*-mutated), PL 11 (*FANCC*-mutated) and CAPAN1 (*BRCA2*-mutated)—have an increased sensitivity to mitomycin C compared to the FA-proficient cell lines (Su 86.86 and MIA Paca-2) [21]. Mutations of *FANCC* and *FANCG* are not detected in Panc 2.03, Panc-1, CFPAC, MIA Paca-2, AsPC-1 and BxPC-3 cells [21]. Since mutations in *TP53* (p53) and *CDKN2A* (p16) have been reported in Panc 2.03, Panc-1, CFPAC, MIA Paca-2, AsPC-1 and BxPC-3 cell lines [23, 24], genetic defects in *TP53* and *CDKN2A* might explain the variation in sensitivity of all these cell lines to resveratrol.

Treatment of normal pancreatic duct cells with resveratrol results in a growth inhibitory effect with an IC_{50} value of 373.89 μM [10]. Thus, the growth inhibition by resveratrol in Panc 2.03 cells is ~17 times greater than in normal pancreatic duct cells, suggesting that resveratrol is selectively toxic against cancer cells. Further investigation into the molecular mechanism of the growth inhibition of this compound is therefore warranted.
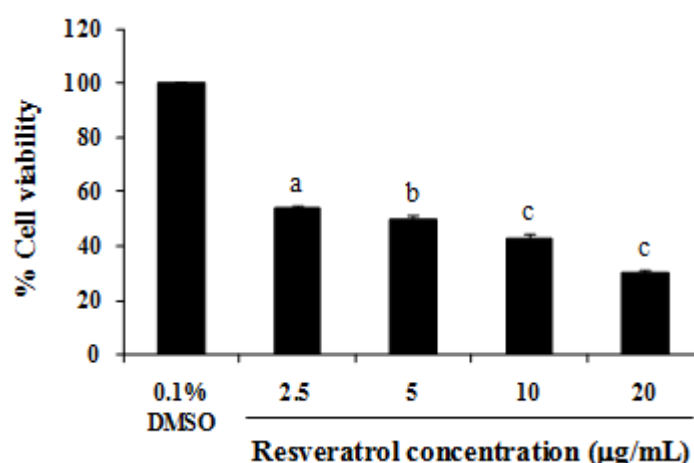

Figure 1. Effect of resveratrol on the growth of Panc 2.03 cells. Cells were treated with 0.1% DMSO or resveratrol at 2.5, 5, 10 and 20 μg/mL for 48 hr. Percent cell viability was determined using sulphorhodamine B assay. Mean ± SE represents 3 different experiments. (a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$ vs. control)

Resveratrol Induces S Phase Cell Cycle Arrest in Panc 2.03 Cells

Perturbations of cell cycle progression can cause inhibition of cell growth. To study the mechanism of resveratrol-induced cell growth inhibition, the effect of resveratrol on cell cycle distribution is determined by flow cytometry. Resveratrol induces significant dose-dependent increase in the percentage of cells in the S phase (from 7% to 46%), while significantly decreasing the percentage of cells in the G0/G1 phase (from 78% to 47%) and the G2/M phase (from 10% to 6%) as compared with the DMSO-treated cells (Figure 2). Our results suggest that resveratrol induces cell cycle arrest in the S phase. The results support previous studies in which the ability of resveratrol to block the S/G_2 transition has been reported with CaCo-2 colon cancer cells [25]. Other investigators, however, have reported arrests in the G_1 phase with Panc-1, AsPC-1, capan-2 and colo 357 pancreatic cancer cells [14, 20]. The effects of resveratrol on cell cycle distribution may therefore vary depending on the source of tumour cells: Panc-1 and Panc 2.03 cells are derived from primary tumours, whereas AsPC-1 is from ascites [26]. Deregulation of cell cycle progression is the hallmark of several human tumours including pancreatic cancer. A variety of mutations in the cell-cycle-regulated genes, including the proto-oncogene K-*ras* and the tumour suppressor genes (*p53*, *p16* and *DPC4*), have been reported in many pancreatic cancer cell lines and patient tissues [13, 26]. It has been reported that Panc-1, Panc 2.03 and AsPC-1 cells contain mutant *p53* and *p16* genes, whereas capan-2 and colo 357 cells contain wild-type *p53* [14, 26]. It has also been reported that K-*ras* is mutated in Panc 2.03, Panc-1 and AsPC-1 cells [26]. Taken together, these results
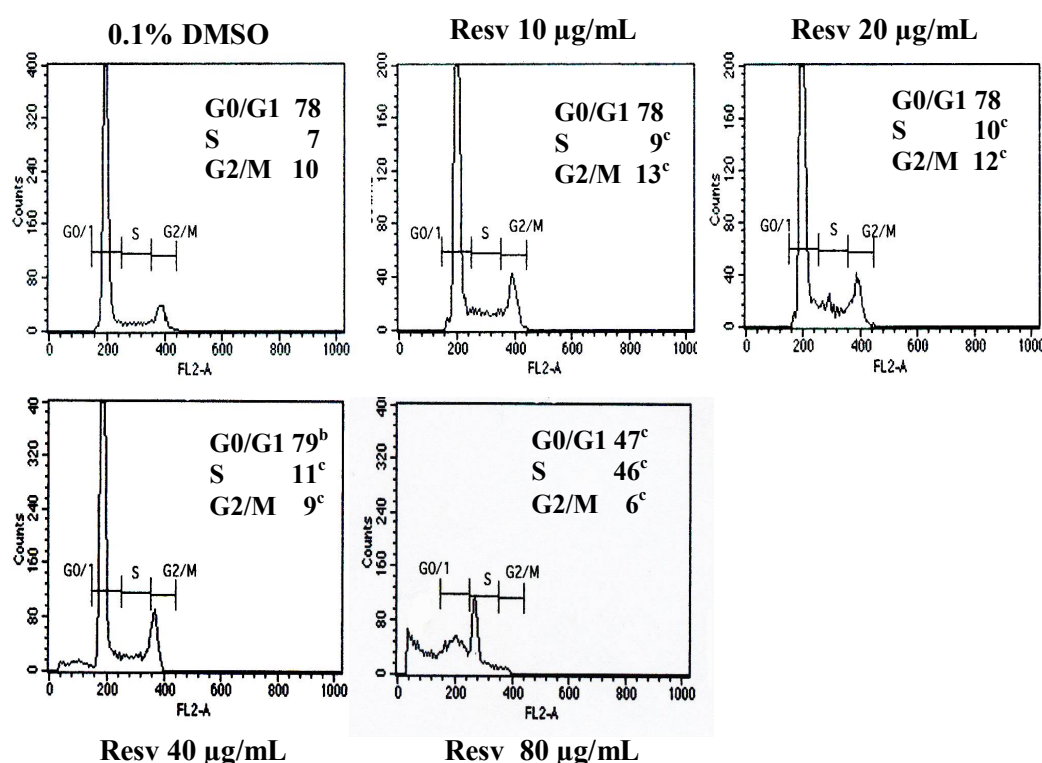

Figure 2. Effects of resveratrol on Panc 2.03 cell cycle distribution. Cells were treated with 0.1% DMSO or various concentrations of resveratrol (Resv) for 48 hr, then stained with GUAVA cell cycle reagent. Percentages of cells in G0/G1, S and G2/M phases of cell cycle were analysed by flow cytometry using CellQuest software. Respective mean ± SE was obtained from 3 independent experiments. (b: $p < 0.01$, c: $p < 0.001$ vs. DMSO-treated cells)

provide evidence that resveratrol induces cell cycle arrest in both *p53* wild-type and *p53* mutant pancreatic cancer cells. The discrepancy in the effects of resveratrol on the cell cycle distribution may be due to differences in the genetic profile of the respective cancer cells.

Apoptosis Induction by Resveratrol in Panc 2.03 Cell Line

The modulation of cell cycle distribution by resveratrol is closely linked to apoptotic cell death (i.e. G0/G1 phase arrest in capan-2, colo 357, Panc-1 and AsPC-1 pancreatic cancer cells) [14, 20]. To determine whether Panc 2.03 cell growth inhibition induced by resveratrol is due to apoptosis, the apoptosis characteristics were determined by several approaches. The typical morphology of apoptotic cells (i.e. cell shrinkage, rounding and membrane blebbing) was observed in the resveratrol-treated Panc 2.03 cells (Figure 3B), whereas no morphological changes were observed in the DMSO-treated cells (Figure 3A). Using 4,6-diamidino-2-phenylindole staining, the morphological changes in the nuclei (i.e. chromatin condensation and nuclear fragmentation, characteristic apoptotic features) were found in the resveratrol-treated cells (Figure 3D) but not in the DMSO-treated cells (Figure 3C). Subsequent visualisation of genomic DNA fragmentation, the hallmark of cell apoptosis in agarose gel electrophoresis, revealed that a 48-hr post-treatment of the Panc 2.03 cells with 20, 40 and 80 µg/mL resveratrol displayed a typical ladder pattern of the inter-nucleosomal DNA fragmentation [27] in a dose-dependent manner (Figure 3E).
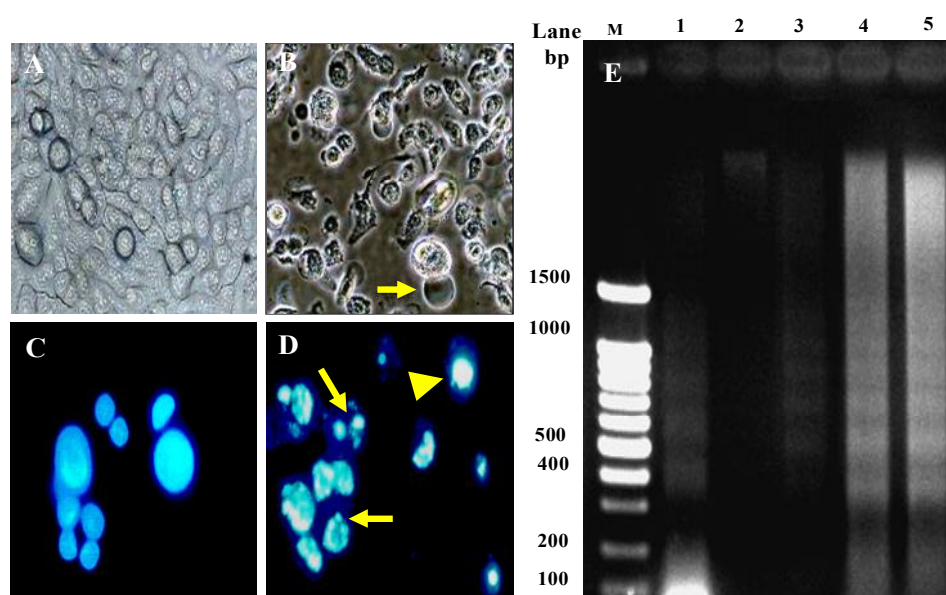

Figure 3. Induced apoptosis of Panc 2.03 cells by resveratrol and effects of resveratrol on morphological changes of Panc 2.03 cells (B and D). After treatment with 0.1% DMSO (A and C) and 40 µg/mL resveratrol (B and D) for 48 hr, cells were photographed by bright field microscope (A and B) or stained with 4,6-diamidino-2-phenylindole and photographed with a fluorescence microscope (C and D). Cells undergoing apoptosis with membrane blebbing are indicated by arrow (B). Nuclei of apoptotic cells show chromatin condensation (D, arrow head) and apoptotic bodies of nuclear fragmentation (D, arrow) (magnification ×400). DNA fragmentation of resveratrol-treated Panc 2.03 cells is evident (E). DNA was extracted from 10 µg/mL etoposide-treated cells (lane 1) or after treatment with 0.1% DMSO, 20, 40 and 80 µg/mL resveratrol (lane 2, 3, 4 and 5) for 48 hr, and then electrophoresed in 1.6% agarose and stained with ethidium bromide. Lane M represents 100 bp DNA markers

The apoptosis induction of Panc 2.03 cells was further confirmed by a flow cytometric analysis using annexin V and propidium iodide (PI) double staining. The percentage of apoptotic cells increased from 13% in 0.1% DMSO-treated cells to 40%, 44% and 53% in resveratrol-treated cells at 20, 40 and 80 µg/mL resveratrol respectively (Figure 4). The apoptosis induction capability of the highest treated group (80 µg/mL) was similar to the positive control, 20 µg/mL etoposide. Taken together, these results demonstrate that the growth inhibition of resveratrol on Panc 2.03 cells is mediated via apoptosis induction.
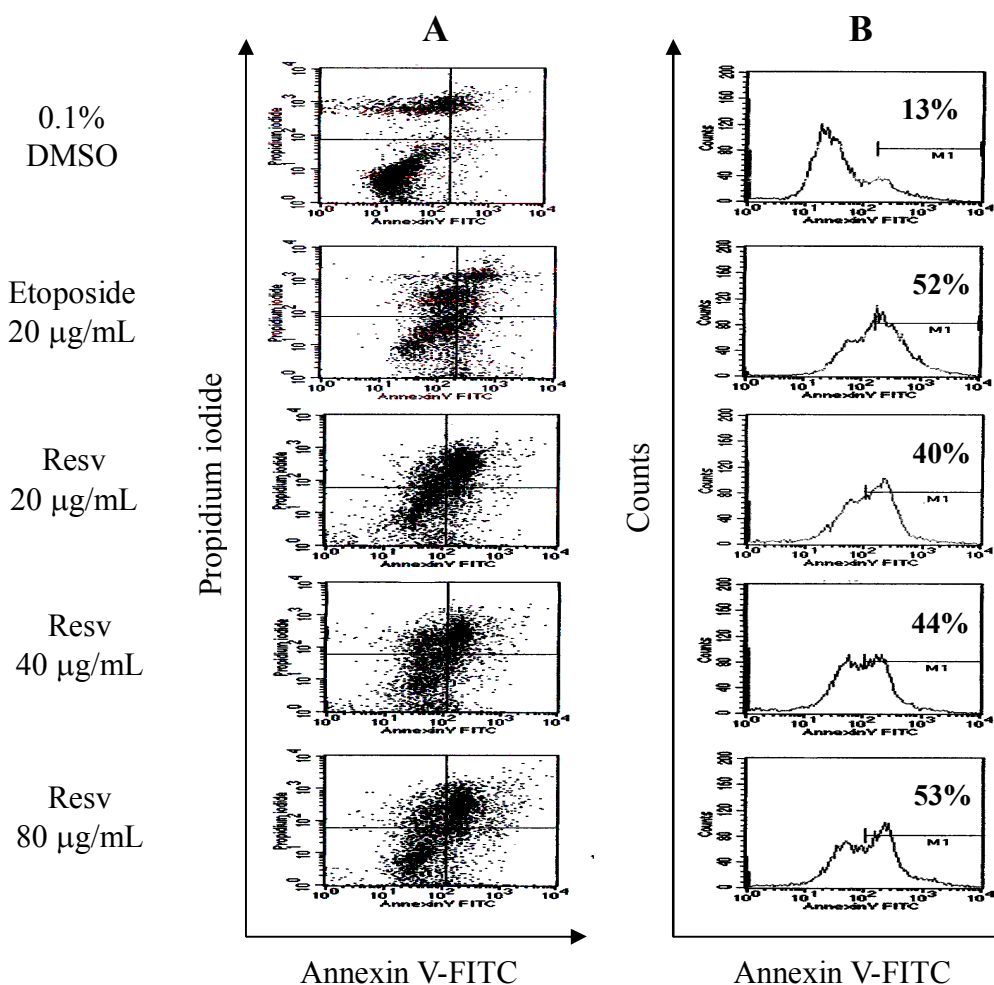

Figure 4. Flow cytometric analysis of cells undergoing apoptosis induced by resveratrol. Cells were cultured for 48 hr in 0.1% DMSO or 20 µg/mL etoposide (positive control) or indicated concentrations of resveratrol (Resv). (A) Apoptotic cells determined by Annexin V-FITC/PI double-staining assay: viable and non-apoptotic cells (lower left corner, annexin V^-/PI^-); early apoptotic cells (lower right corner, annexin V^+/PI^-); late apoptotic cells (upper right corner, annexin V^+/PI^+); necrotic cells (upper left corner, annexin V^-/PI^+). (B) Percentage of apoptotic cells quantified. Values represent average of 3 independent experiments.

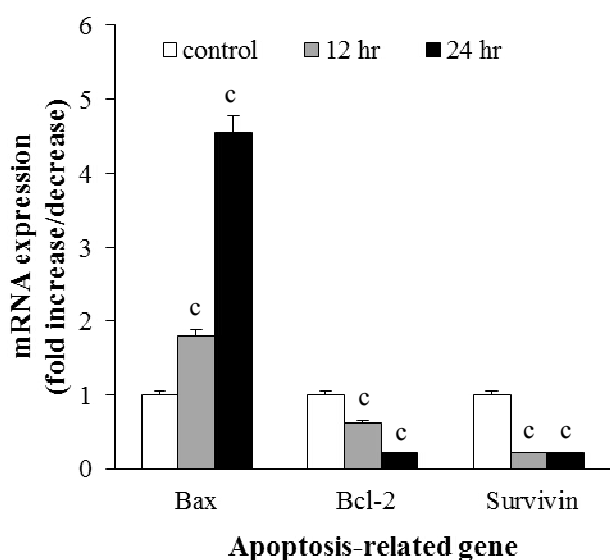**Modulation of Resveratrol on Expressions of Apoptosis-Related Genes and Proteins**

The apoptotic process is highly regulated at different levels through complex interactions of numerous genes and signalling proteins in response to various extracellular and intracellular stimuli. The apoptotic process can be specifically mediated by multiple pathways involving the

mitochondria/cytochrome C (intrinsic pathway), death receptors TNF, CD95 (extrinsic pathway) and granzyme B (cytotoxic T-cell product) [28].

The Bcl-2 family of proteins (Bax and Bcl-2) plays a crucial role in apoptosis by regulating the permeability of the mitochondrial membrane. Bax, in conjunction with other pro-apoptotic proteins, induces apoptosis by forming pores on the mitochondrial membrane, resulting in the leakage of cytochrome C and apoptosis-inducing factor from the membrane. In contrast, the Bcl-2 is an anti-apoptotic protein that helps to stabilise the integrity of the mitochondrial membrane by forming heterodimers with Bax and/or other apoptotic proteins and neutralise their apoptotic activity [29]. Over-expression of Bax can induce apoptosis by suppressing the activity of Bcl-2 [30]. The ratio of Bax/Bcl-2 is therefore a critical factor in determining the apoptotic destiny of cells under certain circumstances [29, 31].

In order to reveal the underlying mechanism of apoptotic execution, the kinetics of apoptosis-related gene expression of Panc 2.03 cells treated with resveratrol at 40 µg/mL were determined at 12-hr and 24-hr post-treatment using real-time PCR analysis. Resveratrol significantly decreased the *Bcl-2* (anti-apoptotic) gene expression, accompanied by a concomitant increase in *Bax* (pro-apoptotic) gene expression, in a time-dependent manner (Figure 5A). Compared to the control cells, *Bcl-2* gene expression was reduced 0.63- and 0.22-fold, whereas *Bax* gene expression was increased 1.79- and 4.56-fold at 12 hr and 24 hr respectively ($p < 0.001$). A marked increase in the *Bax* to *Bcl-2* ratio from 2.84 at 12 hr to 20.73 at 24 hr can be observed (Figure 5B).
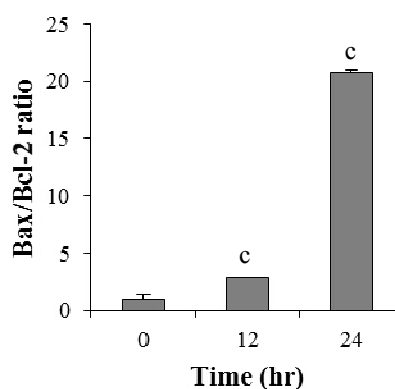

Figure 5. Effects of resveratrol on *Bax*, *Bcl-2* and *survivin* gene expressions of Panc 2.03 cells. Cells were treated with 0.1% DMSO (control) or resveratrol (40 µg/mL). (A) *Bax, Bcl-2* and *survivin* gene expressions were measured using real-time PCR at 12-hr and 24-hr post-treatment. Presented is the fold increase/decrease (mean ± SE) in mRNA expression vs. control, normalised with housekeeping gene glyceraldehyde-3-phosphate, of three independent experiments. (B) Bax/Bcl-2 ratio. (c: $p < 0.001$ vs. control)

The gene expression results, along with the Western blot analysis, demonstrates that the exposure of Panc 2.03 cells to 10, 20, 40 and 80 µg/mL of resveratrol for 48 hr results in a decrease in Bcl-2 protein expression, accompanied by an increase in Bax protein expression, in a dose-dependent manner (Figure 6). Our study reveals that resveratrol, at least in part, induces apoptotic Panc 2.03 cells by modulating the expression of the Bcl-2 family of proteins through the intrinsic (mitochondrial) apoptosis pathway. Moreover, the apoptosis-inducing factor released from the mitochondria can translocate into the nucleus and cause DNA fragmentation via a caspase-independent pathway [9, 28]. In this study, the dose-dependent increase of the apoptosis-inducing factor level in treated Panc 2.03 cells (Figure 6) suggests that the apoptotic execution can also be mediated via a caspase-independent mitochondrial pathway [32].
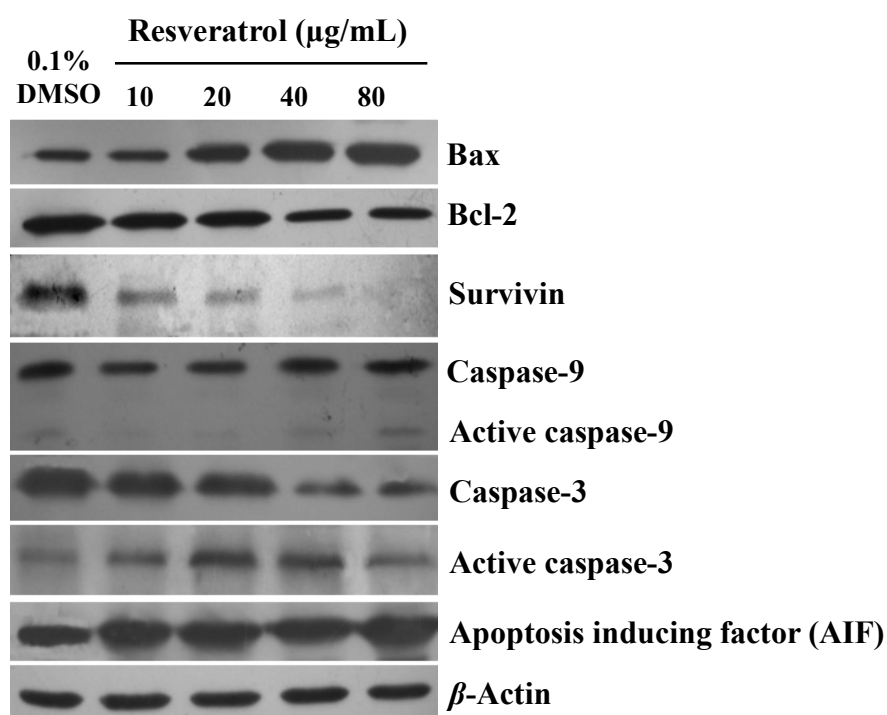

Figure 6. Effects of resveratrol on the expression of apoptosis-related proteins (determined by Western blot analysis) in Panc 2.03 cell. Cells were treated with 0.1% DMSO or the indicated doses of resveratrol for 48 hr.

A recent study [20] shows that resveratrol induces apoptosis in Panc-1, AsPC-1 and BxPC-3 pancreatic cancer cells, which might occur indirectly through the Hedgehog signalling pathway by the down-regulation of Gli1-targeted genes including *Ptc1*, *CCND1* and *Bcl-2*. In addition, resveratrol induces apoptosis by inhibiting miR-21 regulation of Bcl-2 expression in Panc-1, CFPAC-1 and MIA Paca-2 pancreatic cancer cells [10]. Our results likewise show that apoptosis induction of Panc 2.03 cells by resveratrol might be through the down-regulation of Bcl-2 expression.

In the mitochondrial apoptotic pathway, cytochrome C released from mitochondria activates caspase-9 and caspase-3, resulting in DNA fragmentation and apoptotic cell death [33]. Western blot analysis reveals that resveratrol decreases the procaspase-3 level in a dose-dependent manner (Figure 6). The down-regulation of procaspase-3 is accompanied by the up-regulation of active caspase-9 and caspase-3 in the treated groups. Our results suggest that apoptosis induction of Panc

2.03 cells by resveratrol is via the activation of caspase-9 and caspase-3. Our results agree to some extent with a study on capan-2 and colo 357 pancreatic cancer cells, in which resveratrol induces ERK phosphorylation, p21 up-regulation, p53 accumulation and caspase-3 activation, leading to cell cycle arrest and apoptotic cell death [14]. The mechanism of resveratrol-induced apoptosis mediated by cytochrome C release followed by caspase-3 activation has also been reported vis-à-vis other pancreatic cancer cell lines [34].

Survivin is a member of the inhibitor of apoptosis protein family of anti-apoptosis proteins. Since the binding of survivin to active caspase-3 and caspase-7 can inhibit apoptosis [35], the effects of resveratrol on the *survivin* gene and protein expression in Panc 2.03 cells were also examined. The real-time PCR analysis shows that resveratrol induces a dose-related decrease in *survivin* gene expression to a similar extent (about 0.22-fold decrease, $p < 0.001$) after 12-hr and 24-hr post exposure (Figure 5A). The modulation of *survivin* gene expression was subsequently confirmed by a dose-related decrease in survivin protein expression following a 48-hr exposure to 10, 20, 40 and 80 µg/mL of resveratrol (Figure 6). A decrease in survivin level may maintain caspase-3 in an active form, thereby increasing apoptotic cell death [35].

CONCLUSIONS

Our findings demonstrate that resveratrol inhibits the proliferation of Panc 2.03 cells *in vitro* by causing cell cycle arrest at the S phase and inducing apoptosis mediated, at least in part, via a mitochondrial apoptosis pathway in both caspase-dependent and independent manners. The molecular mechanisms involved are the up-regulation of the *Bax/Bcl-2* ratio, the activation of caspase-9 and caspase-3, the down-regulation of survivin and the up-regulation of the apoptosis-inducing factor. The variation in the mechanism of resveratrol-induced cell cycle arrest and apoptosis in different pancreatic cancer cell lines may be dependent on the genetic profile of the cells. Further study of the activity of resveratrol *in vivo* is needed to gain insights into its molecular mechanisms and guide its development for use in chemotherapy of pancreatic cancer.

ACKNOWLEDGEMENTS

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