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Resveratrol Treatment Inhibits Proliferation of and Induces Apoptosis in Human Colon Cancer Cells

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Background: Resveratrol, a natural isolate from plant sources, has a long and important history in traditional Chinese medicine. In the present study we investigated the effect of resveratrol on human colon cancer cell lines.

Material/Methods: We used the Cell Counting kit-8 (CCK-8) for determination of colon cancer cell viability. Apoptosis induction was analyzed using the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA). The siRNA Transfection Reagent kit (Santa Cruz Biotechnology, Inc.) was used for the administration of COX-2 silencer RNA (siRNA) into the colon cancer cells. Primer Express® software for Real-Time PCR ver. 3.0 (Applied Biosystems, Foster City, CA, USA) was used to prepare the primers for RT-PCR.

Results: The results revealed that exposure of colon cancer cells to resveratrol inhibited cell viability. Resveratrol exhibited a significant inhibitory effect on cell viability at 30 μM concentration after 48 h of exposure. We observed that 30-μM doses of resveratrol for 72 h led to 18, 29, and 34% reduction in the viability of HCA-17, SW480, and HT29 cells, respectively. It also significantly induced apoptosis in both of the tested carcinoma cell lines. The population of apoptotic cells in HCA-17 and SW480 cell lines after 48 h of resveratrol treatment was 59.8±4 and 67.2±4%, respectively, compared to 2.3±1% in the control cells. The colon cancer cells exposed to resveratrol showed significantly lower cyclooxygenase-2 and prostaglandin receptor expression. Treatment of colon cancer cells with the inhibitor of cyclooxygenase-2, indomethacin, and administration of silencer RNA for cyclooxygenase-2 also produced similar results.

Conclusions: These findings suggest that resveratrol treatment can be a promising strategy for the treatment of colon cancer.

MeSH Keywords: Apoptosis • Endometrial Neoplasms • Lipoxygenase Inhibitors

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Background

Resveratrol, a natural isolate from plant sources, has a long and important history in traditional Chinese medicine [1]. It has shown promising anticancer activities against breast, prostate, and esophageal cancer cells [2–5]. Resveratrol protects intestinal mucosal barrier in rats with severe acute pancreatitis [6]. The poor prognosis associated with the currently available colon cancer treatment strategies demand the screening of some novel natural anticancer products, like resveratrol [7–9]. Therefore, the present study aimed to investigate the effect of resveratrol on colon cancer cell lines.

Cyclooxygenases (COX) are well known for their role in converting arachidonic acid into prostaglandins [10,11]. The homeostasis in tissues and protection of gastrointestinal tract lining was performed by COX-1. The carcinoma cells, including, pancreatic, colon, breast, hepatic, and gastric cancer, have been found to express higher levels of COX-2 [12–22]. Therefore, onset of colon cancer is indicated by the enhanced production of COX-2 [23]. This is confirmed by the fact that the expression of COX-2 in premalignant adenomas and colorectal carcinomas has been found to be around 45% and 90%, respectively [24]. COX-2 has been shown to play a vital role in colon cancer through the production of prostaglandins and some other factors [25]. Native state arachidonic acid has been reported to induce cellular death without involving any role of prostaglandins [25]. Thus, the conversion of arachidonic acid into prostaglandins through COX-2 overexpression exhibits a significant inhibitory effect on cell apoptosis. COX-2-induced factors influence the development and progression of carcinoma through various cellular processes like apoptosis and angiogenesis [26–28]. It is reported that cyclooxygenase-1 and -2 cause induction of angiogenesis, whereas prostaglandin-2 exhibits an inhibitory effect on apoptosis, promoting Bcl-2 expression [29]. The role of COX-2 can be understood by the fact that its inhibitor, 15-hydroxyprostaglandin dehydrogenase (15-PGDH), inhibits the progression of colon cancer [30,31]. The cyclooxygenase pathway maintains a balance between production and transformation of PGE2 into 15-PGDH [32]. In the present study we investigated the effect of resveratrol on human colon cancer cell lines. The results demonstrated that colon cancer cells exposed to resveratrol showed significantly lower cyclooxygenase-2 and prostaglandin receptor expression.

Material and Methods

Cell culture

HCA-17, SW480, and HT29 colon cancer cell lines were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in RPMI-1640 medium contained in tissue culture flasks. The medium was supplemented with 10% FBS, antibiotics, and 1% glutamine. The cells were cultured at 37°C under a humidified atmosphere of 5% CO_2 and 95% air.

Reagents and chemicals

Resveratrol and dimethyl sulfoxide were obtained from Sigma-Aldrich (St. Louis, MO, USA). The other common chemicals were purchased from Merck (Darmstadt, Germany).

Cell viability assay

The cells at a density of 3×10^5 cells in 100 µl of RPMI-1640 medium containing 10% FBS were distributed into each well of a 96-well plate. The cells were cultured overnight and then treated with different doses of resveratrol for 24 h. Following incubation, the mitochondrial activity in converting 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) to formazan was analyzed using the Cell Counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). The absorbance for each plate was recorded at 450 A° using a spectrometer.

Analysis of apoptotic cell death by flow cytometry

For the purpose of induction of apoptosis in colon cancer cells, a total of 3×10^5 cells in 1 ml of RPMI-1640 medium containing 10% FBS was distributed in each well of a Lab-Tek II Chamber Slide (Nalge Nunc International, Tokyo, Japan). The cells were cultured overnight, treated with different doses of resveratrol, and then incubated for 4 h more. We used the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA) to determine the proportion of apoptotic cells according to the manufacturer's instructions.

Western blot analysis

After treatment with different doses of resveratrol, the colon cancer cells were rinsed 2 times in PBS and lysed in Lysis buffer (50 mMTris-HCl pH 7.4, 137 mM NaCl, 10% glycerol, 100 mM sodium vanadate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% NP-40, and 5 mM cocktail). The concentration of proteins was determined in the cell lysates using BCA method. Electrophoresis was used to isolate the proteins on 10% polyacrylamide gel followed by transfer to a PVDF membrane. The non-specific sites on the membranes were blocked overnight with 5% non-fat dry milk. After washing with TBST, the membranes were incubated with primary antibodies for 12 h. The membranes were again washed with TBST and subsequently incubated with secondary antibodies. Quantity One 1-D Analysis v.4.5.2 software (Bio-Rad) was used to analyze the concentration of COX-2 proteins.

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Administration of COX-2 silencer RNA

The siRNA Transfection Reagent kit (Santa Cruz Biotechnology, Inc.) was used for the administration of COX-2 silencer RNA (siRNA) into the colon cancer cells. Briefly, the cells were allowed to attain proper confluence and then we administered the siRNA for COX-2. The medium was then exchanged with fresh medium after incubation for 24 h. The cells were incubated in the replaced medium for 48 h, harvested, and analyzed for apoptotic death using trypan blue exclusion assay. Inhibition of the expression of COX-2 was analyzed using Western blot analysis.

Quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR)

Primer Express® software for Real-Time PCR ver. 3.0 (Applied Biosystems, Foster City, CA, USA) was used to prepare the primers for RT-PCR based on sequences present in GenBank. The primers used were obtained from Hokkaido System Science (Hokkaido, Japan). SYBR-Green real-time PCR Master Mix-Plus (Toyobo, Osaka, Japan) and an Applied Biosystems 7300 real-time PCR system (Applied Biosystems) were used for the purpose of RT-PCR according to the manufacturer's instructions.

Animals and tumor xenograft assay

Twenty female 8-week-old mice were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). The animals were fed with irradiated chow and had free access to tap water. The animals were randomly assigned to 2 groups of 10 animals each: the resveratrol group and the untreated group. Both groups were administered 3×10^4 HCA-17 cells intraperitoneally in Matrigel (Basement Membrane Matrix, High Concentration; BD Biosciences). The mice in the resveratrol group received 30-μM doses of resveratrol dissolved in DMSO daily for 1 month and those in the untreated group were given the same volume of DMSO. On every alternate day up to the 2-month time period, the volume of the tumor was measured using a caliper and the observations were compared between the 2 groups. The mice were sacrificed at 61 days after administration; tumors were extracted, weighed, formalin-fixed, and embedded in paraffin.

Ethics statement

This study was approved and performed in accordance with the guidelines for animal use and care of Shanghai Laboratory Animal Center (Ref. no: 2014-133SLAC).

Statistical analysis

Data are expressed as means ± standard deviations of samples. Comparison among groups was performed using the unpaired *t* test. One-way analysis of variance with the Bonferroni post-test was used for the analysis of the data obtained. In all cases, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of cell proliferation by resveratrol treatment

We observed that exposure of HCA-17, SW480, and HT29 colon cancer cell lines to resveratrol inhibited proliferation in a dose- and time-dependent manner. The cells were exposed to different doses of resveratrol (0, 10, 20, 30, or 50 μM) to investigate its effect on cell viability. We observed that 30-μM doses of resveratrol for 72 h led to 18%, 29%, and 34% reduction in the viability of HCA-17, SW480, and HT29 cells, respectively (Figure 1A, 1B).

Induction of apoptosis by resveratrol in colon cancer cells

The results revealed that exposure of HCA-17 and SW480 carcinoma cell lines to resveratrol caused significant induction of apoptosis in both of the tested cell lines (Figure 1C). Examination of the cell cultures treated with 10-, 20-, and 30-μM doses of resveratrol for 72 h showed induction of apoptosis in 23.5±2, 39.7±2, and 67.2±4% of cells, respectively (Figure 1D). Exposure of SW480 cells to 10-, 20-, and 30-μM doses of resveratrol caused apoptosis in 21±2, 35.6±2, and 59.8±4% of cells, respectively (Figure 1C, D).

COX-2 and PGE2 are highly expressed in colon carcinoma cells

Comparison of the expression level of COX-2 in the colon carcinoma and normal (CCD-18Co) cell lines showed a significantly higher level in the carcinoma cells (Figure 2A). The expression level of PGE2 was also markedly higher in HCA-17, SW480, and HT29 carcinoma cell lines compared to the CCD-18Co normal cell line (Figure 2B).

Inhibition of COX-2 and PGE2 expression by resveratrol

We analyzed the effect of resveratrol on the expression of COX-2 in colon carcinoma cell lines after 72 h. We observed that resveratrol treatment had a concentration-dependent inhibitory effect on the expression of COX-2 in HCA-17, SW480, and HT29 cell lines. Among various doses of resveratrol (0, 10, 20, 30, or 50 μM), the inhibition of COX-2 was significant at 30 μM dose after 72 h (Figure 2C). In addition, the expression of PGE2 in HCA-17, SW480, and HT29 cells was also inhibited by resveratrol treatment after 72 h of treatment (Figure 2D).

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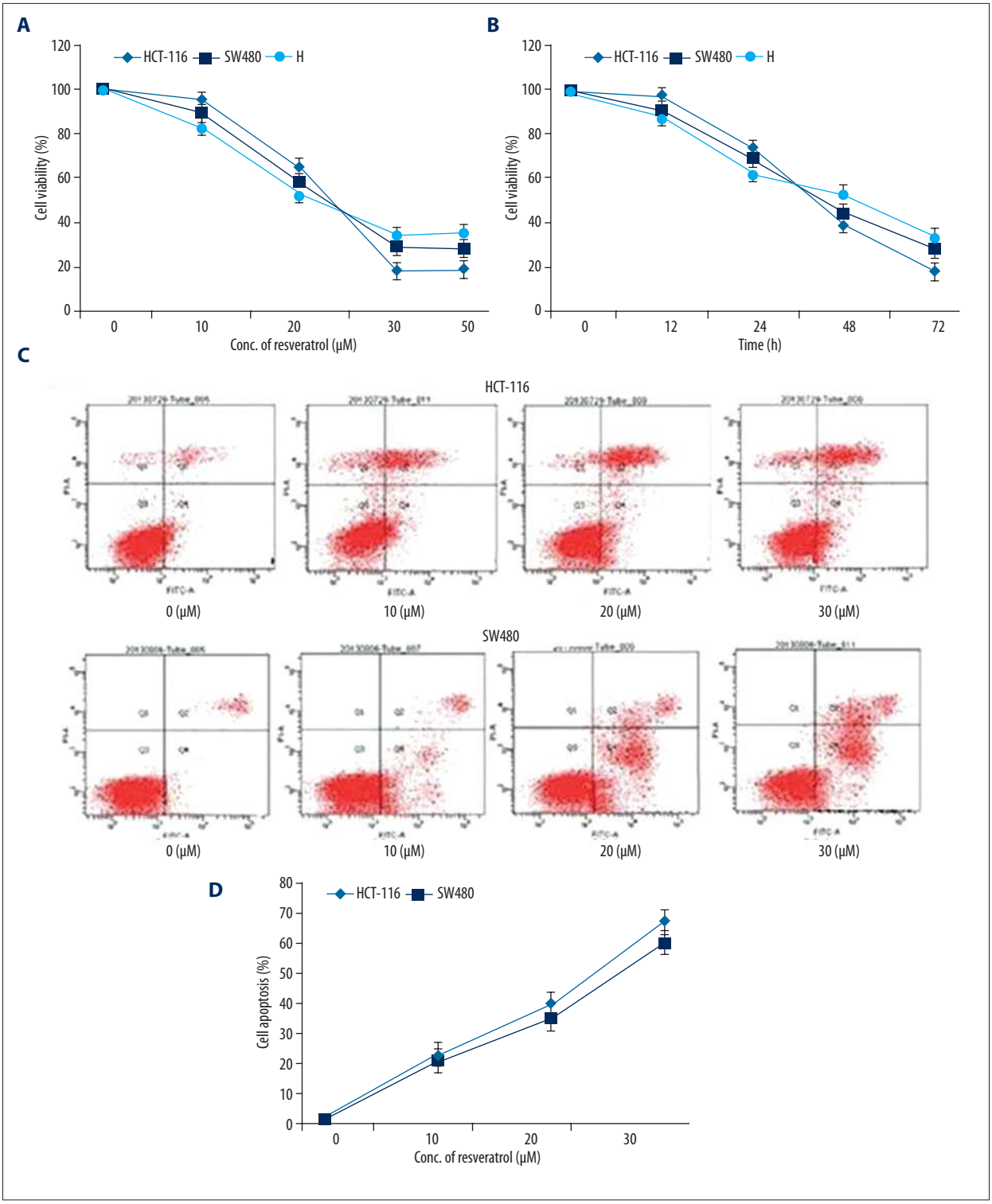

Figure 1. Inhibition of HCA-17 and SW480 cell proliferation and induction of apoptosis by resveratrol. (**A**) HCA-17 and (**B**) SW480 cells were treated with different doses of resveratrol for 72 h and then analyzed for cell viability. (**C, D**) HCA-17 and SW480 cells were analyzed for induction of apoptosis after treatment with different doses of resveratrol.

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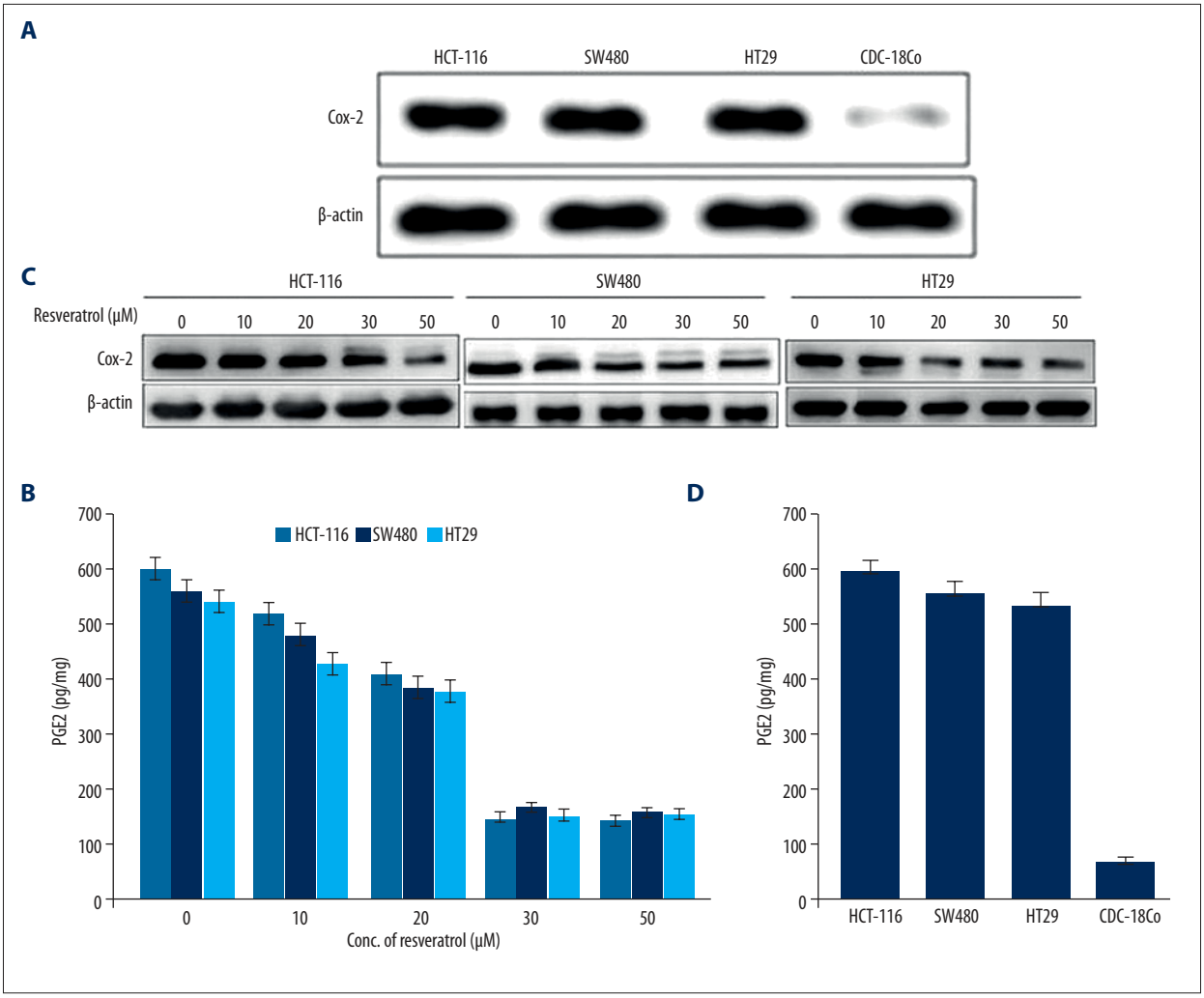

Figure 2. Inhibition of the basal levels of COX-2 and PGE2 expression in colon cancer cells by resveratrol treatment. (**A, B**) Expression of COX-2 and PGE2 in the cells before treatment with resveratrol. (**C, D**) Effect of resveratrol on the expression of COX-2 and PGE2 in HCA-17, SW480, and HT29 carcinoma cells and normal (CCD-18Co) cell lines.

Indomethacin inhibits growth and induces cell death in colon cancer cells

Treatment of the colon carcinoma cell lines, HCA-17, SW480, and HT29 with indomethacin showed similar results for inhibition of cell proliferation as that of resveratrol. Analysis of apoptosis in the cells treated with indomethacin for 48 h showed induction of apoptosis to the same extent as that of resveratrol (Figure 3A, 3B). These findings suggest that resveratrol-induced inhibition of proliferation and induction of apoptosis in colon cancer cells involves inhibition of COX-2 expression. Administration of COX-2 silencer RNA in HCA-17, SW480, and HT29 cells led to the inhibition of cell proliferation and induction of apoptosis after 48 h of exposure (Figure 3C).

Effect of resveratrol on PGE2-induced cell proliferation of colon cancer cells

Colon carcinoma cells treated with PGE2 for 48 h showed a markedly higher rate of proliferation than in control cells (Figure 4). However, resveratrol treatment in HCA-17 and SW480 cells at a dose of 30 μM for 72 h inhibited PGE2-mediated promotion of colon cell proliferation (Figure 4). Treatment of colon carcinoma cells with indomethacin suppressed proliferation in both the PGE2-treated and PGE2-untreated cells. However, treatment with a combination of resveratrol + indomethacin synergistically decreased the PGE2-stimulated cellular proliferation of HCA-17 and SW480 cells (Figure 4).

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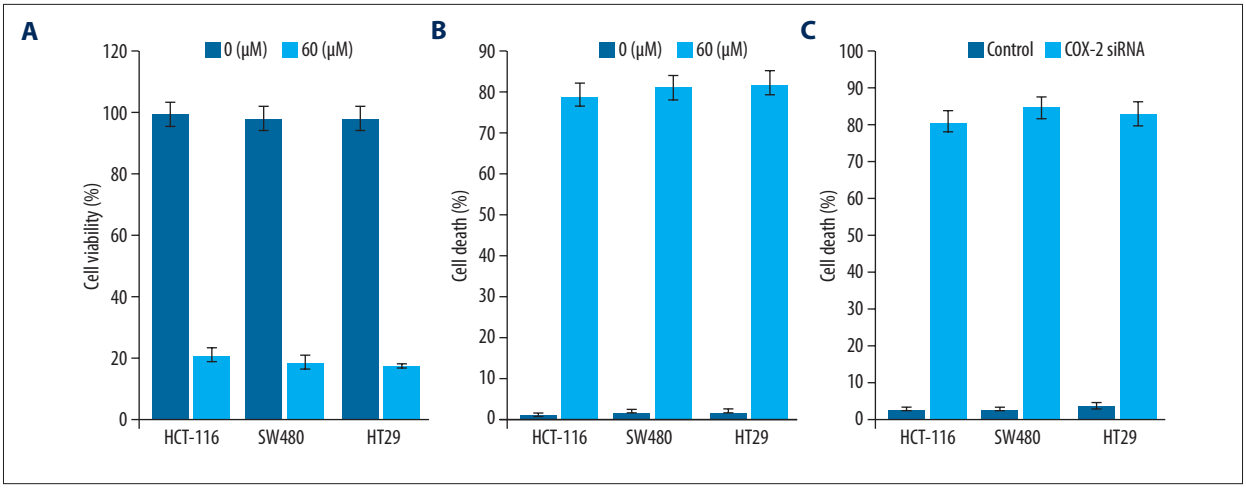

Figure 3. Effect of 48-h indomethacin treatment and COX-2 inhibition on HCA-17, SW480, and HT29 cell proliferation and induction of cell death. (**A, B**) Indomethacin treatment for 48 h reduced cell viability and induced cell death. (**C**) COX-2 siRNA induced cell death in HCA-17, SW480, and HT29 carcinoma cells.

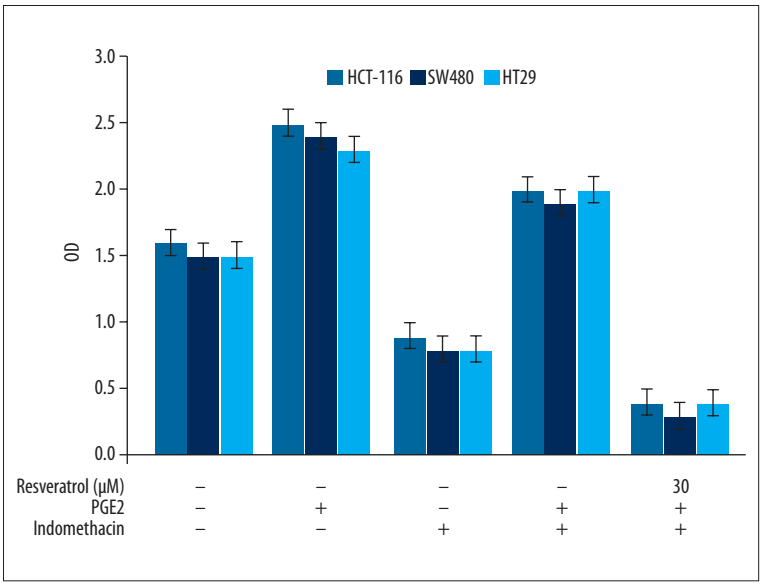

Figure 4. Effect of resveratrol on inhibition of PGE2-induced cell proliferation in colon cancer cells.

Effect of resveratrol on up-regulation of PGE2 receptors in colon cancer cells

Exposure of HCA-17, SW480, and HT29 cell lines to resveratrol for 48 h induced reduction in the expression of EP4 (Figure 5A, 5B). However, the exposure of HCA-17, SW480, and HT29 cells to EP4 agonist led to a marked promotion of cell proliferation ($P<0.01$) (Figure 5). However, when EP4 agonist-treated cells were exposed to resveratrol, cell proliferation was significantly reduced ($P<0.001$).

Discussion

The overexpression of COX-2 leads to tumor invasion, angiogenesis, reduced host immunity, and development of apoptotic resistance by tumor cells [33]. PGE2, a metabolic product of COX-2, mediates most of the protumorigenic effects, and its high concentration creates a microenvironment conducive to tumor growth. Therefore, discovery of a potent COX-2 inhibitor may be a promising therapeutic strategy for the treatment of colon cancer.

The results of the present study revealed significant inhibition of colon cancer cell growth *in vitro* and *in vivo* by resveratrol treatment. Resveratrol induces apoptotic cell death and induces inhibition of COX-2 expression and PGE2 production.

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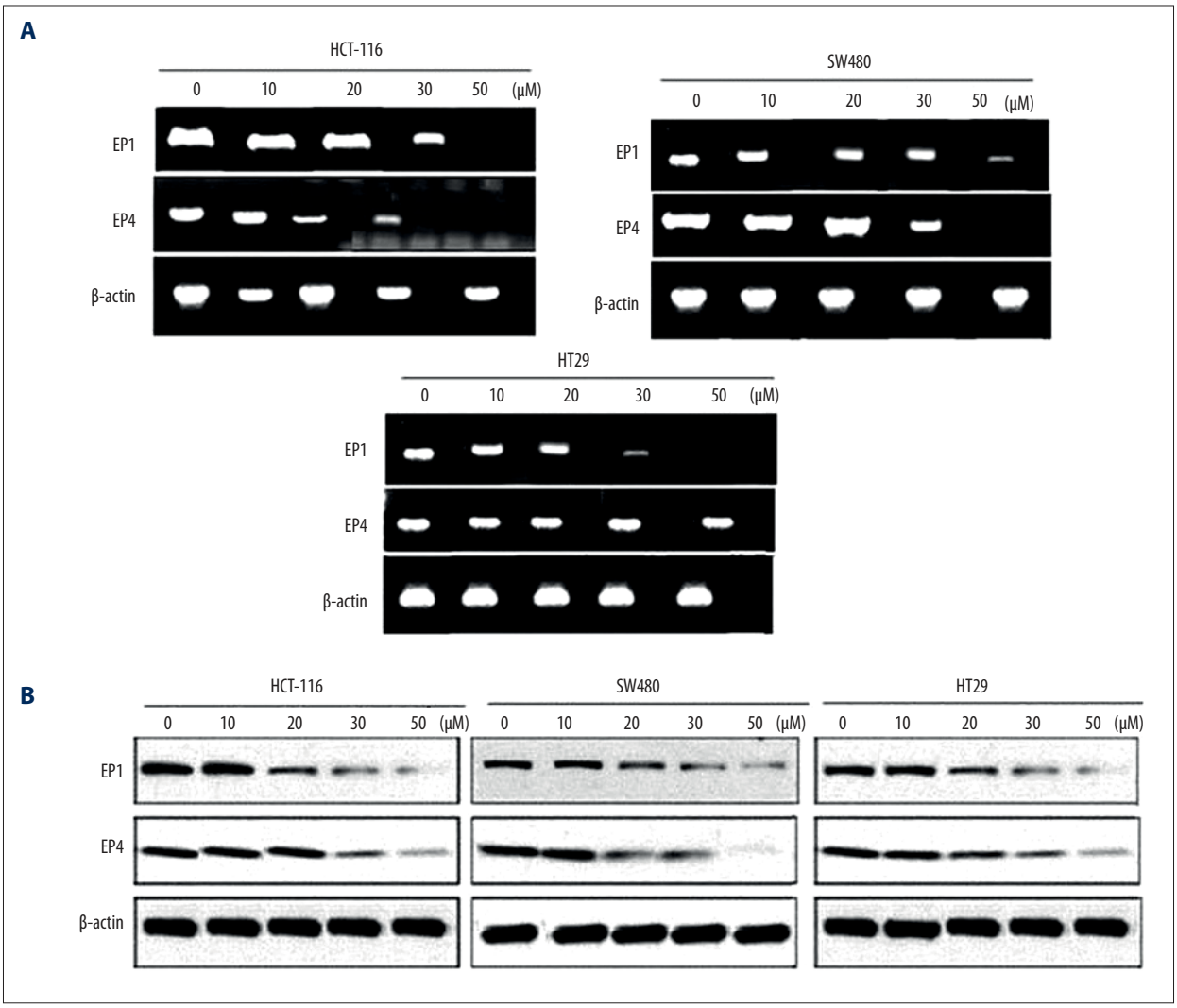

Figure 5. Effect of various concentrations of resveratrol on inhibition of expression of PGE2 receptors EP1 and EP4 in HCA-17, SW480, and HT29 cells using (**A**) RT-PCR and (**B**) Western blot assay.

COX-2 is overexpressed by colon cancer cells, and it appears that the inhibition of COX-2 by resveratrol may be responsible for the apoptosis of these cells. This is proved by the fact that the colon cancer cells treated with the pan-COX inhibitor, indomethacin, showed a significant reduction in cell proliferation and enhanced rate of apoptosis. Transfection of colon cancer cells with COX-2 siRNA also led to similar results.

It is well known that PGE2 exerts its multiple effects through 4 G protein-coupled receptors – EP1, EP2, EP3, and EP4 [34] – which can stimulate epithelial cell growth and invasion and promote cellular survival [35,36]. We observed that SW480 cells express the PGE2 receptors, EP1 and EP4, and that the expression of EP1 and EP4 was reduced when cells were treated with resveratrol *in vitro*. These data suggest that the inhibition of the EP1 and EP4 levels by resveratrol may contribute to the inhibition of tumor cell growth and induction of apoptosis of colon cancer cells.

This assumption is based on the findings that PGE2 receptors are coupled to the GαS, and ligand binding has been reported to increase cyclic AMP levels, leading to the activation of PKA and Akt [37]. Akt and PKA activation can mediate prosurvival pathways through the inactivation of proapoptotic proteins [38,39]. Our results are consistent with the report that PGE2 protects gastric mucosal cells *in vitro* from ethanol-induced apoptosis via EP1 and EP4 activation [40]. The inhibitory effect of resveratrol on colon cancer cell proliferation through the inhibitory effect on EP1 or EP4 was further confirmed by treating the cells with EP4 agonist. We found that the treatment of SW480cells with EP4 agonist (PGE1 alcohol) resulted in enhanced cell proliferation, and that EP4 agonist-induced cell proliferation was inhibited by the treatment of cells with resveratrol. This observation further supports the concept that the inhibition of PGE2 receptors by resveratrol may have contributed to the inhibition of proliferation and induction of apoptosis in colon cancer cells.

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To determine if resveratrol can inhibit the growth of colon cancer cells *in vivo*, the colon tumor xenografts grown in athymic mice were analyzed for the expression of COX-2, PGE2, and PGE2 receptors. The results revealed that the inhibition of tumor xenograft growth in athymic nude mice fed a diet supplemented with resveratrol was associated with the inhibition of COX-2 and PGE2 expression along with decrease in the levels of PGE2 receptors, EP1, EP3, and EP4. Our results suggest that the protective effects of resveratrol on the growth of colon cancer cells *in vivo* are induced through the inhibition of PGE2 and PGE2 receptors.

Conclusions

Resveratrol can be a promising chemotherapeutic agent for the treatment of colon cancer through inhibition of COX-2 and PGE2 expression.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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