Paeonol exerts an anticancer effect on human colorectal cancer cells through inhibition of PGE_2 synthesis and COX-2 expression

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Abstract. Cyclooxygenase-2 (COX-2) and its metabolite prostaglandin E_2 (PGE_2) can potentially affect most of the events in cancer development, including promotion of proliferation, resistance to apoptosis, angiogenesis, immune suppression and invasion. However, worldwide attention has predominantly centered on the cardiovascular toxicity of selective COX-2 inhibitors. Paeonol is a major active extract from the root bark of *Paeonia suffruticosa* Andrews with anti-inflammatory, anti-oxidant, anti-allergic, anti-oxidation and antitumor effects. In the present study, we investigated the underlying mechanisms of paeonol in inducing apoptosis and aimed to ascertain whether its antitumor effect is associated with a reduction in COX-2 expression and a decrease in the levels of PGE_2 in colorectal cancer cells. We observed that paeonol inhibited cell proliferation and induced apoptosis in a dose- and time-dependent manner in colorectal cancer cells, which was associated with a reduction in COX-2 expression and PGE_2 synthesis. Treatment with the selective COX-2 inhibitor, celecoxib, or transient transfection of colorectal cancer cells with COX-2 siRNA, also inhibited cell proliferation and induced apoptosis. Western blot analysis showed that paeonol inhibited the activation of NF-κB, an upstream regulator of COX-2, and its translocation to the nucleus. Treatment with increasing doses of paeonol led to increased expression of pro-apoptotic factor Bax and decreased expression of anti-apoptotic factor Bcl-2. Caspase-3 and caspase-9 were activated, and paeonol induced loss of mitochondrial membrane potential, suggesting that the apoptosis induced by paeonol was mediated by mitochondrial pathways. In addition, paeonol significantly suppressed tumor growth in a xenograft tumor mouse model in a dose-dependent manner. Our findings indicate that paeonol exerts an antitumor effect on human colorectal cancer cells by inhibiting PGE_2 production and COX-2 expression. We expect that paeonol may replace selective COX-2 inhibitors due to their toxic effects, and may offer a new strategy for the therapy of colorectal cancer.

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Introduction

Colorectal cancer is a common malignant tumor of the digestive system, and the mortality associated with this disease is ranked second highest among all malignant neoplasms in industrialized countries (1). The role of cyclooxygenase-2 (COX-2) in colorectal cancer development and progression has been extensively studied. COX-2 is overexpressed in large and high-grade dysplasia adenomas, and COX-2 overexpression is associated with carcinogenesis, progression, invasion, metastasis and a poor prognosis (2-5). Deregulation of COX-2 expression leads to an increased abundance of prostaglandin E_2 (PGE_2), which can potentially affect most of the events in cancer development, including proliferation, resistance to apoptosis, angiogenesis, immune suppression and invasion (6,7). Because of its important role in tumor formation, progression, invasion and metastasis, COX-2 is considered as a promising target for cancer therapy (8,9).

There is increasing evidence demonstrating that inhibition of expression of COX-2 has antitumor activity against gastrointestinal carcinoma (10). It has been confirmed that selective COX-2 inhibitors are effective in preventing adenoma recurrence and reducing the incidence of colorectal cancer to some extent (11). However, previous research has revealed that unexpected cardiovascular side effects result when selective COX-2 inhibitors are used in the long term (12). Consequently, the development of more effective and low-toxicity selective COX-2 inhibitors is an important area of study.

Paeonol (2-hydroxy-4-methoxyacetophenone), a major active extract from the root bark of *Paeonia suffruticosa* Andrews, possesses a number of biological activities, including anti-inflammatory (13,14), anti-oxidant (15), anti-angiogenic (16), anti-allergic (17) and anti-oxidation (18). Paeonol has been shown to exhibit anti-proliferative effects and apoptosis-inducing activities in various types of tumor cell lines *in vitro* and *in vivo* (19,20). Our previous study also revealed that paeonol induced apoptosis in colorectal cancer cells (21). Here, in the present study, we further investigated the underlying mechanisms of paeonol in inducing apoptosis

and whether its antitumor effect is associated with reduction in COX-2 expression and a decrease in the levels of PGE_2 in colorectal cancer cells.

Materials and methods

Materials. Colorectal cancer cell lines HCT116, SW620 and LoVo were obtained from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Paeonol was purchased from Natura Pharmaceutical Co., Ltd. (purity >99.0%; Ningbo, China). DMEM/F-12 medium, fetal bovine serum (FBS), penicillin-streptomycin, pancreatin and glutamine and the bicinchoninic acid (BCA) protein assay kit were purchased from Beyotime Institute of Biotechnology (Suzhou, China). The Annexin V-FITC/propidium iodide (PI) apoptosis kit was purchased from Roche Ltd. (Shanghai, China). Celecoxib, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and Rhodamine 123 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 2X Taq PCR Master Mix was obtained from Tiangen Biotech Co., Ltd. (Beijing, China). Primers for human COX-2 and β-actin were designed by Sangon Biotech Co., Ltd. (Shanghai, China), and the sequences were as follows: forward, 5'-AAT GAG TAC CGA AAA TTC-3' and reverse, 5'-CAT CTA GTC CGG ACC GGG AAG-3' for COX-2; forward, 5'-ACC ACA GTC CAT GCC ATC AC-3' and reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3' for GAPDH. COX-2 siRNA was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA). The primary antibodies against human COX-2, Bax, Bcl-2, caspase-3, caspase-9, NF-κB, IKKα, IκBα and actin were obtained from Cell Signaling Technology (Beverly, MA, USA). The secondary antibodies were purchased from Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, China). All other chemicals were of reagent grade and were obtained from commercial sources. Animals were maintained according to animal care guidelines from the Institutional Animal Care and Use Committee of Wuhan University. The study was approved by the Institutional Review Board of Renmin Hospital of Wuhan University.

Cell culture. All the cell lines were cultured in a 5% CO_2 and 95% humidified air atmosphere at 37˚C in DMEM/F-12 medium supplemented with heat-inactivated 10% FBS, 1% antibiotics (100 IU penicillin and 100 μg/ml streptomycin).

Cell viability assay. MTT assay was used to analyze the viability of the cell lines after test agent treatment. Briefly, all cancer cell lines were seeded into 96-well plates ($6.0x10^3$ cells/well) and allowed to attach overnight. After cellular adhesion, the medium was replaced with fresh medium supplemented with various concentrations of test agents and further cultivated for the indicated periods. Test agents were added to the culture medium at various indicated concentrations. The control culture received only the culture medium. Following further incubation, MTT was added at a concentration of 5 mg/ml, and the cells were incubated for another 4 h at 37˚C. The medium was discarded, and DMSO was added to dissolve the MTT formazan crystals. The absorbance reading of each well was determined using a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm. The wells without test agents and the free cells (culture medium alone) were used as background. The cell growth inhibitory rates were defined as the relative absorbance of the treated vs. the untreated cells.

Cell apoptosis assay. To quantify apoptosis, cells were stained with Annexin V and PI using the Annexin V-FITC/PI Apoptosis kit according to the manufacturer's instructions. Briefly, colorectal cancer cells were cultured in 6-well plates with medium for 24 h. The cells were then treated for a further 48 h with the test agents. After treatment, the cells were washed twice with cold phosphate-buffered saline (PBS) following treatment and resuspended in 195 μl Annexin V-FITC binding buffer. Annexin V-FITC (5 μl) was added and mixed gently, and the cells were incubated for 15 min at room temperature in the dark. The cells were then centrifuged at 1,000 x g for 5 min and gently resuspended in 190 μl Annexin V-FITC binding buffer. Following this, 10 μl PI staining solution was added and gently mixed. The cells were kept on ice in the dark and immediately subjected to flow cytometry (FACScan; BD Biosciences, San Diego, CA, USA).

Determination of PGE_2 production. To quantify PGE_2 in the cell culture supernatant, a competitive ELISA was performed according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA). Briefly, colorectal cancer cells were cultured in the 6-well plates with medium for 24 h. The cells were then treated for a further 48 h with various concentrations of paeonol. After treatment, the LoVo cell supernatant was diluted 1:4 for analysis. The samples were tested in duplicate for each of three independent experiments. Optical density at 405 nm was analyzed using a SpectraMax M2 plate reader. The concentration of PGE_2 was determined using SoftMax Pro 5.4.2 plate reader software (Molecular Devices, Sunnyvale, CA, USA). A standard concentration curve was generated for each independent experiment.

COX-2 siRNA synthesis and transfection. Colorectal cancer cells ($2x10^5$ in 2 ml of DMEM/F-12 without antibiotics) were plated in 6-well plates. After 24 h, the human-specific COX-2 siRNA mix with Lipofectamine 2000 was overlaid on the cells according to the manufacturer's protocol. After 48 h of transfection, cells were harvested for the cell viability assay, the cell apoptosis assay, reverse transcription-polymerase chain reaction (RT-PCR) analysis and western blot analysis.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was measured by Rhodamine 123 staining. Colorectal cancer cells were cultured in 6-well plates and allowed to attach overnight. After cellular adhesion, the cells were then treated for a further 48 h with various indicated concentrations of the test agents. Cells were harvested and washed twice with PBS and then incubated with 20 μl Rhodamine 123 staining solution at 37˚C in the dark for 30 min, and then washed twice with PBS and centrifuged at 500 x g for 10 min. Finally, the absorbance was determined using a spectrofluorometer at an excitation wavelength of 505 nm and an emission wavelength of 534 nm.

RT-PCR analysis. Total RNA was isolated using TRIzol reagent and 1 μg RNA was used as a template for the synthesis of cDNA using the RevertAid First Strand cDNA synthesis kit (Fermentas, Waltham, MA, USA) according to the manufacturer's instructions. PCR analysis was performed in a final volume of 25 μl using PCR Master Mix. PCR products were separated in 1.5% agarose gel, stained with ethidium bromide and images were captured.

Western blot assay. Protein expression levels were analyzed by western blot analysis. Briefly, colorectal cancer cells were seeded in 6-well plates at a density of $2x10^5$ cells and were then incubated overnight at 37˚C before treatment. After cells were treated with various indicated concentrations of the test agents for 48 h, the cells were washed with PBS and lysed with lysis buffer, and incubated at 4˚C for 1 h. The extracts were cleared by centrifugation at 13,000 rpm for 20 min at 4˚C. The concentration of protein was determined using a BCA protein assay kit according to the manufacturer's instructions. Protein was loaded at a concentration of 40 μg/lane, separated on a 12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel, and then transferred onto a nitrocellulose membrane using a wet transfer system. Next, the membrane was blocked with 10% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST, pH 8.0) and then incubated with the primary antibodies: COX-2, Bax, Bcl-2, caspase-3, caspase-9, NF-κB, IKKα, IκBα and actin overnight at 4˚C. The appropriate horseradish peroxidase (HRP) conjugated secondary antibodies were used at 1:1,000 for all antibodies. Positive antibody reactions were detected with the enhanced chemoluminescence system and Hyperfilm X-ray film.

Xenograft tumors in mice. Male BALB/c nude mice, 4-5 weeks of age, obtained from the Center of Experimental Animals of Wuhan University, were used in all of the experiments. LoVo cells, $5x10^6$, suspended in 100 μl PBS, were subcutaneously inoculated into the lower right flank of the nude mice. After 7 days, the nude mice were randomly divided into four groups (n=6 in each group): the control group received 100 μl PBS (intragastric administration); the three paeonol groups were intragastrically administered paeonol (100, 200 and 400 mg/kg/day). There were no statistical differences among the sizes of all the groups. Drugs were administrated daily by gavage for 11 day. On day 12, all mice were sacrificed and the tumor xenografts were removed and weighed. The tumor growth inhibitory rate was calculated using the following formula: Inhibition rate (%) = (1 - mean test tumor weight/mean control tumor weight) x 100.

Statistical analysis. All continuing values are expressed as the mean ± SD. Student's t-test was used for comparison of the values between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Paeonol inhibits human colorectal cancer cell proliferation. The three human colorectal cancer cell lines were treated with 30 mg/l paeonol, and the viability of cells was assessed by MTT assay from 24 to 72 h. After treatment, the proliferation of these cell lines was significantly inhibited, especially in the LoVo cells (Fig. 1A). Furthermore, the growth rate of LoVo cells was greatly decreased by incubation with 60 and 120 mg/l paeonol (Fig. 1B). The viability of LoVo cells treated with paeonol was decreased in a dose- and time-dependent manner.

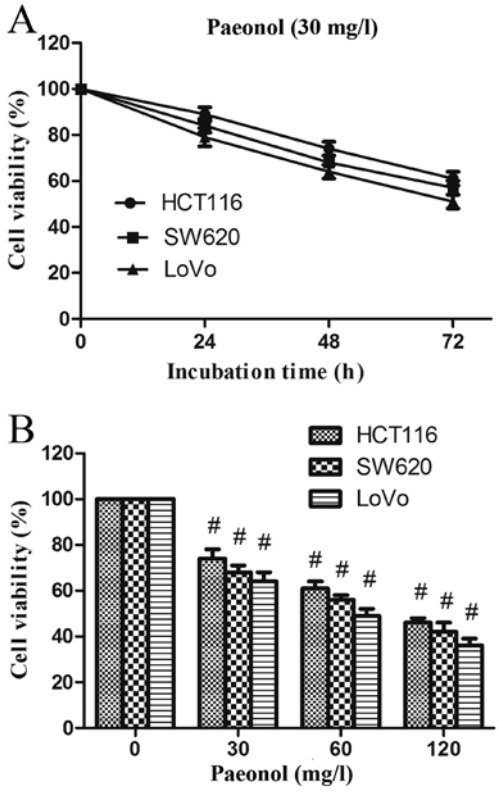

Figure 1. Paeonol inhibits colorectal cancer cell proliferation *in vitro*. (A) Cell viability of three colorectal cancer cell lines HCT116, SW620 and LoVo treated with 30 mg/l paeonol for 0, 24, 48 and 72 h. (B) Cell viability of colorectal cancer cell lines treated with various concentrations of paeonol for 48 h ($^{\#}$P<0.05 vs. 0 mg/l).

Paeonol induces cell apoptosis in colorectal cancer cells. To determine whether the growth-inhibitory effect of paeonol is related to the induction of apoptosis, colorectal cancer cells treated with paeonol for 48 h were analyzed using flow cytometric analysis. The proportion of apoptotic cells increased from 15.4 to 38.6% in a dose-dependent manner (Fig. 2). The percentage of apoptotic cells treated with paeonol was significantly higher compared with that in the control group (P<0.01), indicating that paeonol may inhibit the growth of colorectal cancer cells by inducing apoptosis.

Paeonol downregulates the expression of COX-2 in colorectal cancer cells. COX-2, overexpressed in various types of cancers, plays an important role in tumor formation, progression, invasion and metastasis. To elucidate the interaction between COX-2 and paeonol, LoVo cells were exposed to 0, 30, 60 and 120 mg/l paeonol for 48 h, and the expression of COX-2 was assessed using western blot analysis. Paeonol treatment was associated with reduced expression of COX-2 (Fig. 3A). The expression of COX-2 in cells treated with 30 mg/l paeonol was observed to be lower than that in the controls. Treatment

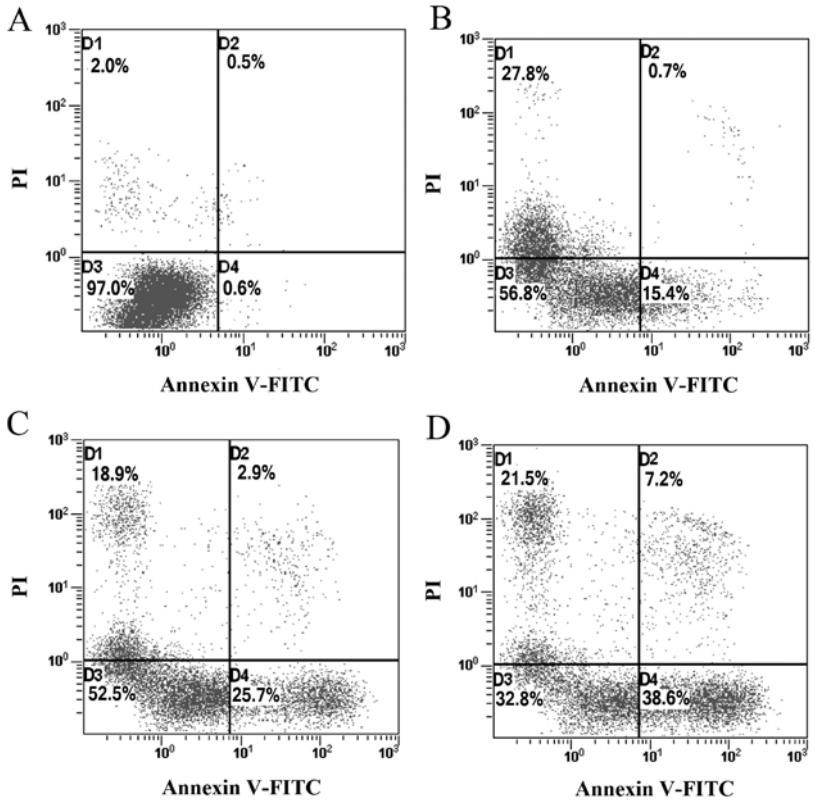

Figure 2. Paeonol induces apoptosis in LoVo cells. LoVo cells were treated with the indicated concentrations of paeonol for 48 h, and the effect of paeonol on cell apoptosis was analyzed by flow cytometry: (A) 0, (B) 30, (C) 60 and (D) 120 mg/l paeonol.

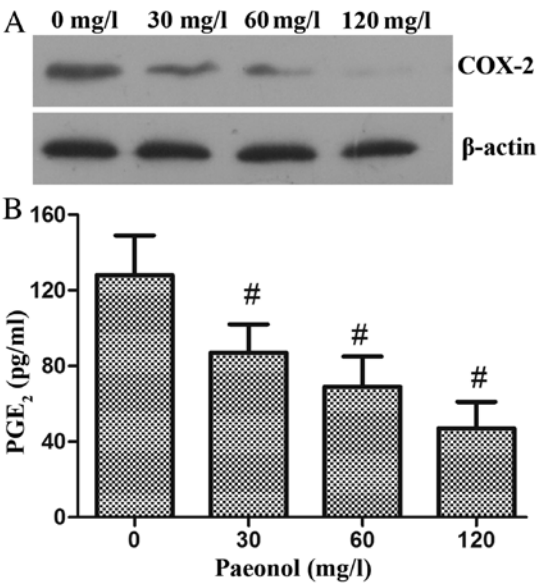

Figure 3. Paeonol reduces cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) synthesis in LoVo cells. (A) The levels of COX-2 were determined in cell lysates using western blot analysis. Treatment of LoVo cells with paeonol resulted in downregulation of COX-2 expression. (B) Dose-dependent effect of paeonol on the levels of PGE_2 in the LoVo cell supernatant. #P<0.05 vs. 0 mg/l.

with 120 mg/l paeonol led to a further decrease, indicative of a dose-dependent decrease in COX-2 expression.

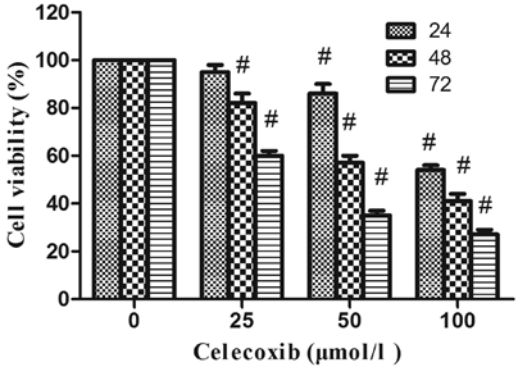

Figure 4. Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, inhibits cell proliferation and induces cell apoptosis in colorectal cancer cells. The viability of LoVo cells treated with the indicated concentrations of celecoxib for 0, 24, 48 and 72 h is shown. Treatment with celecoxib caused a dose- and time-dependent inhibition of cell proliferation. #P<0.05 vs. 0 mg/l.

Paeonol reduces PGE_2 synthesis in colorectal cancer cells. As the COX-2 metabolite, PGE_2 has been implicated in COX-2-mediated effects including cancer cell proliferation and apoptosis, we further assessed the levels of PGE_2 in the paeonol-treated cells. Treatment with paeonol for 48 h resulted in a significant reduction in the production or synthesis of PGE_2 in the LoVo cells in a concentration-dependent manner, suggesting that paeonol-induced reduction in PGE_2 production is associated with the inhibitory effect of paeonol on COX-2 expression, inhibition of cell proliferation, and induction of cell apoptosis (Fig. 3B).

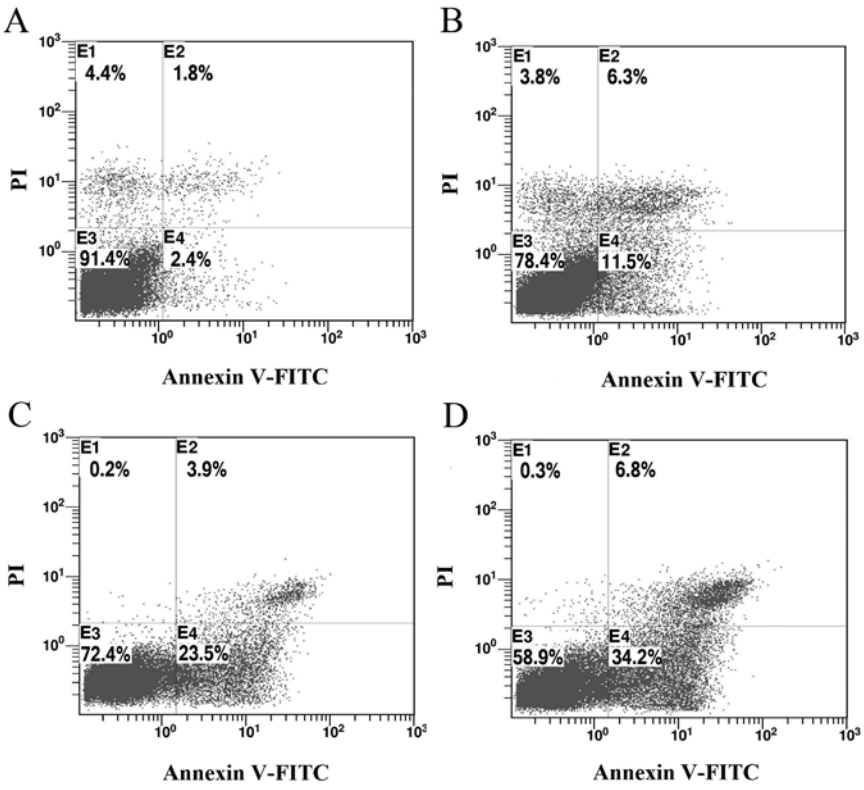

Figure 5. Celecoxib induces apoptosis in LoVo cells. LoVo cells were treated with the indicated concentrations of paeonol for 48 h, and Annexin V/PI staining was performed: (A) 0, (B) 25, (C) 50 and (D) 100 μmol/l paeonol.

Celecoxib, a selective COX-2 inhibitor, inhibits colorectal cancer cell proliferation and induces cell apoptosis. This experiment was performed to explore whether the antitumor effect of paeonol on colorectal cancer cells is mediated through its inhibitory effect on COX-2 expression. For this purpose, the viability of LoVo cells was assessed by MTT assay after treatment with various concentrations of celecoxib (0, 25, 50 and 100 μmol/l), a well-known inhibitor of COX-2, from 24 to 72 h. Treatment of the cells with celecoxib resulted in a dose- and time-dependent reduction in the cell viability of LoVo cells as compared with non-celecoxib-treated controls ($P<0.05$) (Fig. 4). Celecoxib also could induce cell apoptosis. After treatment with celecoxib for 48 h, the proportion of apoptotic cells increased from 11.5 to 34.2% in a dose-dependent manner (Fig. 5). These data suggest that the inhibition of COX-2 expression is linked to the inhibition of cell proliferation and induction of cell apoptosis.

siRNA knockdown of COX-2 leads to inhibition of cell proliferation and induction of cell apoptosis. We further verified the role of COX-2 in cell proliferation and apoptosis through siRNA knockdown of COX-2 in the colorectal cells and examined whether it would lead to the inhibition of cell proliferation and the induction of cell apoptosis. After 48 h of transfection, the expression of COX-2 was analyzed by RT-PCR and western blot analysis. The mRNA expression and protein expression levels of COX-2 were significantly decreased in the COX-2 siRNA group compared with the control siRNA group. We assessed the potential effects of downregulation of COX-2 on the proliferation in LoVo cells (Fig. 6A). The transfection of LoVo cells with COX-2 siRNA resulted in a significant reduction in the cell proliferation of LoVo cells after 48 h as compared to that of the control siRNA-transfected LoVo cells (Fig. 6B). We also analyzed the effect of COX-2 siRNA on cell apoptosis in LoVo cells using Annexin V-FITC/PI staining. After 48 h of transfection, the proportion of apoptotic cells was significantly increased in the COX-2 siRNA group compared with that of the control siRNA group (Fig. 6C).

Paeonol inhibits the activation of NF-κB in colorectal cancer cells. NF-κB is an upstream regulator of COX-2; therefore, we further examined whether paeonol could also decrease the activation of NF-κB in colorectal cancer cells.

For this purpose, after treatment with paeonol (0, 30, 60 and 120 mg/ml) for 48 h, LoVo cells were harvested and whole cell lysates and nuclear lysates were prepared.

The results of the western blot analysis indicated that paeonol reduced the nuclear translocation of NF-κB/p65 in a dose-dependent manner. In addition, treatment with paeonol resulted in the downregulation of IKKα and degradation of IκBα, which was responsible for the inactivation of NF-κB and its translocation to the nucleus (Fig. 7A).

Paeonol induces cell apoptosis through activation of the mitochondrial pathway in colorectal cancer cells. To examine the mechanism of apoptosis induced by paeonol in the colorectal cancer cells, we analyzed mitochondrial features of the intrinsic apoptotic pathway. After treatment with paeonol (0, 30, 60 and 120 mg/l) for 48 h, western blot analysis was performed to assess the protein expression levels of Bax,

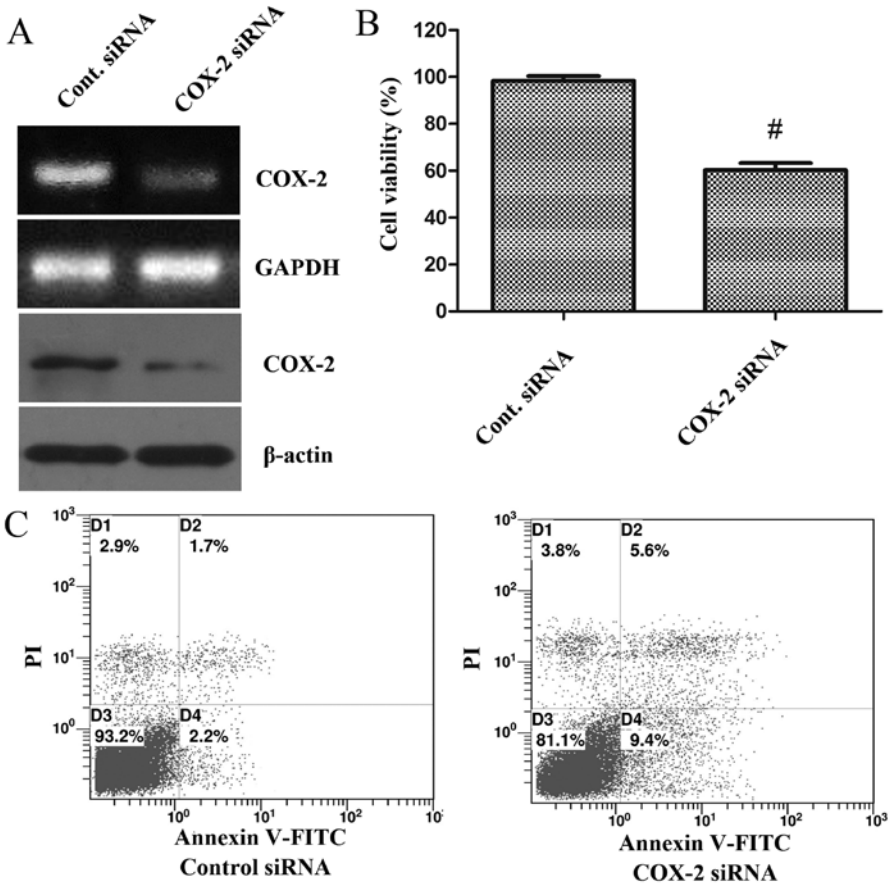

Figure 6. siRNA knockdown of cyclooxygenase-2 (COX-2) leads to inhibition of cell proliferation and induction of cell apoptosis. (A) COX-2 expression was analyzed by PT-PCR analysis and western blotting in LoVo cells and COX-2 siRNA-transfected LoVo cells. (B) Forty-eight hours after transfection, the proliferation of LoVo cells was significantly inhibited. #P<0.05 vs. control siRNA-transfected LoVo cells. (C) The apoptosis-inducing effect was analyzed by flow cytometry after 48 h of transfection.

Bcl-2, caspase-3 and caspase-9 (Fig. 7B and C). Treatment with increasing doses of paeonol led to increased expression of Bax and decreased expressions of anti-apoptotic Bcl-2, and the stimulation of caspase-3 and caspase-9 activity, which is considered as a hallmark of the apoptotic process.

Paeonol decreases mitochondrial membrane potential in colorectal cancer cells. Disruption of mitochondrial integrity is a critical step occurring in cells undergoing apoptosis, and a decreasing mitochondrial membrane potential is related to mitochondrial dysfunction. Moreover, loss of mitochondrial membrane potential plays a vital role in mitochondrial-mediated apoptosis. After treatment with various concentrations of paeonol for 48 h, the fluorescence intensity of Rhodamine 123 was significantly decreased in the LoVo cells, suggesting that paeonol treatment of colorectal cancer cells induced apoptosis through the mitochondrial apoptosis pathway (Fig. 7D).

Antitumor effect of paeonol on colorectal cancer cells in vivo. After the investigation of apoptosis induction in LoVo cells *in vitro*, we further evaluated the antitumor effect of paeonol. LoVo cells were subcutaneously inoculated into the lower right flank of nude mice. After 7 days, the nude mice were treated with different concentrations of paeonol via oral gavage every day. Tumor weight was obtained at the end of the experiment. Tumor weight in the paeonol groups was significantly decreased (P<0.05 vs. control). The tumor growth inhibitory rates in the paeonol groups were 22.35, 31.32 and 36.54%, respectively (Table I).

Discussion

Colorectal cancer is the second most common cause of cancer-related mortality in the world. On a world-wide basis, there were more than 1,200,000 new cases in 2012, with more than 600,000 deaths (22). Although much progress in treatment and diagnosis has been achieved in recent years, the survival rate and survival period of colorectal cancer patients have not significantly improved. Of patients with newly diagnosed colorectal cancer, 15-25% have metastatic disease, which is usually lethal (23). Therefore, additional research is urgently required to investigate new treatment options and innovative therapeutic strategies.

Previous studies have revealed that paeonol exhibits anti-proliferative effects in various tumor cell lines *in vitro* and *in vivo* (19-21). However, the underlying mechanisms remain unknown. In the present study, our results showed that paeonol effectively inhibited HCT116, SW620 and LoVo cell proliferation, particularly in LoVo cells, in a dose- and time-dependent manner *in vitro*. In the xenograft tumor-bearing nude mouse

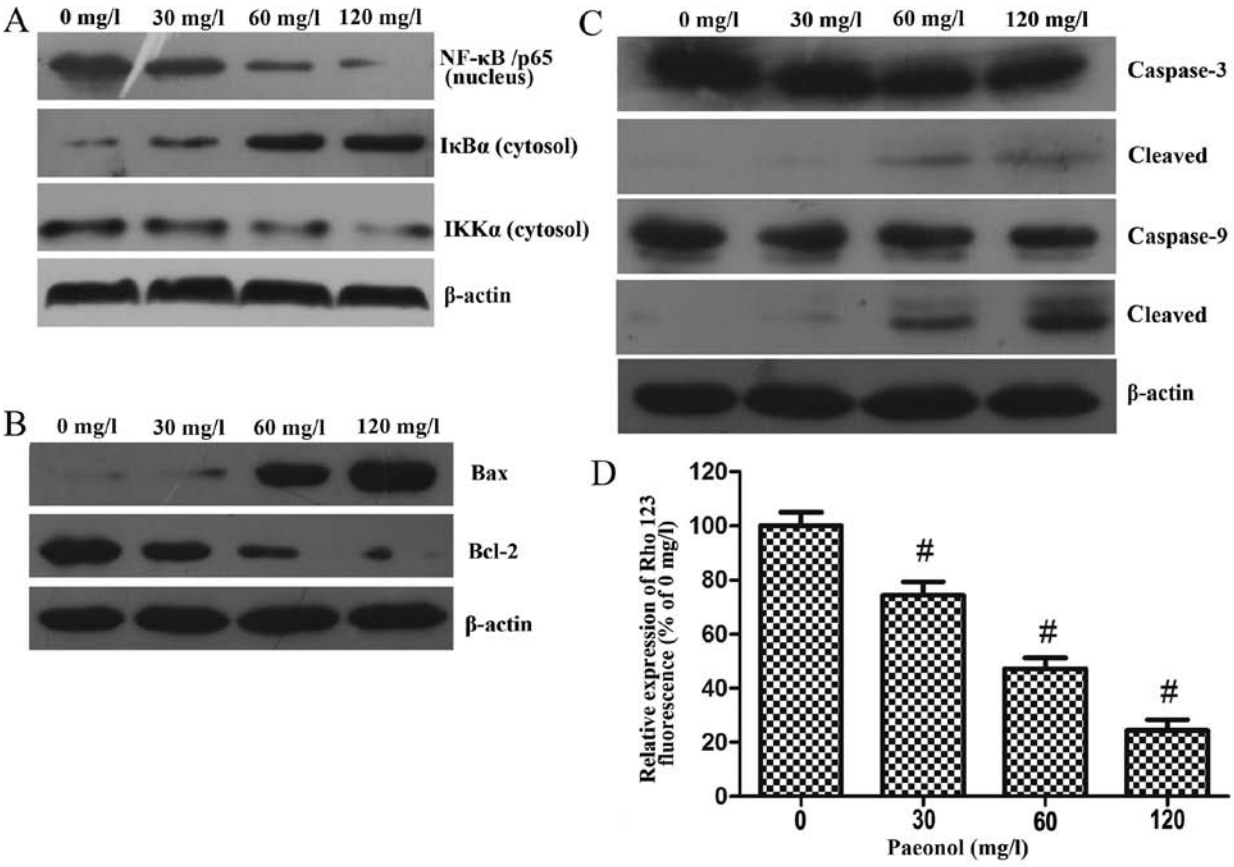

Figure 7. Effects of paeonol treatment on the activation of NF-κB, expression of pro-apoptotic and anti-apoptotic elements, and the level of mitochondrial membrane potential. (A) Treatment of LoVo cells with paeonol decreased the levels of NF-κB/p65 and IKKα while inhibiting the degradation of IκBα. (B) Western blot assay was performed to assess the protein expression levels of Bax and Bcl-2 after treatment with paeonol for 48 h. (C) Paeonol-induced caspase activation. Activation of caspase-3 and caspase-9 was detected by western blot analysis. (D) Paeonol reduced the Rhodamine 123 fluorescence intensity in LoVo cells treated with paeonol for 48 h, suggesting paeonol reduces mitochondrial membrane potential. #P<0.05 vs. 0 mg/l.

Table I. Inhibitory effect of paeonol on LoVo cell xenotransplanted tumors in BALB/c mice.

Group	n	Tumor weight (g)	Inhibitory rate (%)
Control	6	0.613±0.062	-
100 mg/kg paeonol	6	0.476±0.044[a]	22.35
200 mg/kg paeonol	6	0.421±0.071[a]	31.32
400 mg/kg paeonol	6	0.389±0.054[a]	36.54

Data are presented as mean ± SD. The inhibitory rate (%) = (1 - mean of tumor weight of test group/mean of tumor weight of control) x 100. [a]P<0.05 vs. control.

model, paeonol was revealed to have significant anticancer effect. It also revealed that paeonol resulted in apoptosis of treated cells in a dose-dependent manner. These results indicated that paeonol may inhibit the proliferation of colorectal cancer cells by activating the apoptotic signaling pathway.

Apoptosis, a tightly regulated signaling process that involves the coordination of both anti-apoptotic and pro-apoptotic proteins, is vital for anti-carcinogenesis (24). The pro-apoptotic Bcl-2 family members, such as Bax and Bcl-2, are essential for the initiation of mitochondrial dysfunction during apoptosis. The results showed that the expression of pro-apoptotic factor Bax was markedly upregulated in the paeonol treatment group. However, anti-apoptotic factor Bcl-2 was significantly reduced. Moreover, the paeonol-induced apoptotic response involved caspase-3 and caspase-9 activation in the colorectal cancer cells. Paeonol also induced loss of mitochondrial membrane potential in the LoVo cells. This suggests that Bcl-2 inhibited Bax activity, which reduced mitochondrial membrane potential, leading to caspase-3 upregulation and cell apoptosis (25). Together these results indicate that paeonol induced apoptosis in colorectal cancer cells by activating the mitochondrial-mediated apoptosis pathway.

COX-2, the inducible enzyme that regulates PGE_2 synthesis, is frequently overexpressed in various types of cancers (26,27). COX-2 and PGE_2 play a central role in orchestrating multiple events of cancer invasion, metastasis and tumor development (28-30). Thus, COX-2 is considered as a promising target for cancer therapy (8,9). Three controlled randomized trials (PreSAP, APC and Approve) were launched to evaluate the efficacy and safety of cyclooxygenase-2 inhibitors (COXIBs) in preventing the recurrence of sporadic colorectal adenomas. The data showed that the use of COXIBs was associated with a significant reduction

in the risk of adenoma recurrence, particularly of advanced adenomas in all of these studies (31-33). However, the cardiovascular toxicity associated with the use of COXIBs limits their use in healthy individuals. Therefore, the search for novel and low-toxic inhibitors of COX-2 may provide a better option for the treatment of colorectal cancer and this may prove to be a better strategy for its prevention or treatment. In the present study, paeonol inhibited the expression of COX-2 and the production of PGE_2. These results indicated that the effects of paeonol on cell grow inhibition and apoptosis were associated with the inhibion of COX-2 expression and PGE_2 synthesis. This was supported by evidence that treatment of LoVo cells with celecoxib, a selected COX-2 inhibitor, resulted in a reduction in the cell viability and an increase in cell apoptosis. Similar effects were noted when the LoVo cells were transfected with COX-2 siRNA. Protein encoded by COX-2 genes is a type of oncogenic protein, which could catalyze the conversion of arachidonic acid to PGE_2. Research has confirmed that overexpression of COX-2 promotes cell proliferation by weakening the anti-proliferative effect of transforming growth factor-β (TGF-β) (34). A previous study demonstrated that COX-2 is a regulatory factor in the Bcl-2 upstream sequences through the phosphatidylinositol 3-kinase (PI3K) signaling pathway, and eventually inhibits the apoptosis of cancer cells (35). It has also been confirmed that COX-2 inhibits the apoptosis of cancer cells by inducing the mutation of P53 and weakening the apoptotic signal mediated by Fas protein (36). These findings indicate that paeonol induces apoptosis by suppressing the expression of COX-2, which leads to downregulation of Bcl-2 and upregulation of Bax.

NF-κB is a family of dimeric transcription factors that regulate a wide spectrum of biological processes, including inflammation, immune responses, cell proliferation and apoptosis (37-39). Moreover, NF-κB downstream effectors including COX-2, Bax and Bcl-2 are key mediators of apoptosis and cell cycle arrest. In the present study, treatment of paeonol resulted in the downregulation of IKKα and degradation of IκBα, eventually leading to the inactivation of NF-κB and its translocation to the nucleus. As previously mentioned, paeonol also affected the NF-κB-regulated apoptosis-related proteins including Bax and Bcl-2.

In conclusion, the results from this study suggest that paeonol exhibits a marked antitumor effect. One of the antitumor mechanisms of paeonol may be that its inhibition of NF-κB and COX-2 leads to reduced proliferation and induction of apoptosis, connected with caspase-dependent mitochondrial dysfunction. These results provide a rationale to continue research on paeonol, and further mechanism-based studies are required. We expect that paeonol may replace selective COX-2 inhibitors due to their toxic effects, and may become a new strategy for the therapy of colorectal cancer.

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