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Original Research Article

Paeonol enhances TRAIL-induced apoptosis of human lung cancer cells by upregulating death receptors-4 and 5 via ROS-JNK/ERK-CHOP signaling

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Abstract

Purpose: *To study the anti-proliferative potential of paeonol against lung cancer cells, and investigate its mechanism of action.*
Methods: *Cell viability after paeonol treatment was determined with 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT) assay, while paeonol- and TRAIL-mediated apoptosis was assayed using flow cytometry. Western blotting was used to assay the protein expression levels of phosphorylated JNK and ERK1/2, as well as protein expressions of pro-apoptotic factors/death receptors. 2',7'-Dichlorodihydrofluorescein diacetate (H2DCFDA) staining and flow cytometry were used to monitor paeonol-induced reactive oxygen species (ROS) in the cells.*
Results: *Paeonol treatment markedly reduced the proliferations of H1975 and BGC823 cells ($p < 0.05$). In H1975 and BGC823 cells, paeonol/TRAIL combination increased apoptosis to 88.43 and 87.21 %, respectively ($p < 0.05$). The levels of death receptor 4 (DR4) and death receptor 5 (DR5) were increased significantly by paeonol, relative to the control ($p < 0.05$). Paeonol also reduced the levels of decoy receptor-1 (DcR1) and decoy receptor-2 (DcR2), and increased the expression of CHOP ($p < 0.05$). The protein expression levels of survivin, Bcl-2, cFLIP and Bcl-xL were decreased, while protein levels of caspase3, caspase-8 and caspase-9 were upregulated by paeonol. Moreover, paeonol significantly upregulated p-ERK and p-JNK in H1975 and BGC823 cells, and also increased ROS levels, when compared to control ($p < 0.05$).*
Conclusion: *Paeonol exerts anti-proliferative potential on lung cancer cells through upregulation of death receptors, activation of JNK/ERK-CHOP pathway and generation of ROS. Therefore, paeonol has a therapeutic potential for the management of lung cancer.*

Keywords: *Paeonol, Proliferation, Reactive oxygen species, Apoptosis, Tumour necrosis factor*

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INTRODUCTION

Lung cancer is a leading cause of mortality among various types of cancers globally, notwithstanding huge advancement in chemotherapy and diagnostic techniques [1]. Non-small cell lung cancer (NSCLC), the primary type of lung carcinoma, is diagnosed in 85 % of patients, whereas small cell lung cancer (SCLC) accounts for about 15 % of lung cancer

cases [2]. The 5-year survival of patients with NSCLC is still very low (15 %), despite the use of radiation and chemotherapy [3]. Chemotherapeutic compounds generally exert their anticancer effects through activation of apoptosis. Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) is a member of the super-family of tumour necrosis factors. Studies have revealed that TRAIL specifically targets carcinoma cell growth via activation of apoptosis without reduction of normal cell viability [4].

It has been demonstrated that TRAIL exerts its effect by interacting with DR4 and DR5 death receptors in the membrane via ligands attached to it [5]. In addition, TRAIL influences the activities of decoy receptor (DcR)-1, DcR2 and the receptor protein osteoprotegerin [6]. The interaction amongst TRAIL, DR4 and DR5 links to Fas-associated death domain results in activation of caspases, leading to apoptosis [7]. The selectivity of TRAIL in inducing carcinoma cell apoptosis makes it a preferred anticancer candidate in cancer therapy. Unfortunately, some carcinoma cells have developed resistance to the apoptotic effect of TRAIL via different mechanisms [8]. Studies have revealed down-regulated DR4 and DR5 expressions and enhanced decoy receptor levels in carcinoma cells resistant to TRAIL [8, 9]. The level of FLICE-like inhibitory protein [cFLIP(L)] and suppressors of apoptosis are up-regulated in tumor cells resistant to TRAIL [10].

Compounds isolated from plants sensitize carcinoma cells to TRAIL-mediated apoptosis [11,12]. Paeonol is a low molecular weight compound obtained from Cortex Moutan, a Chinese herbal plant [13]. The plant is known for its anti-inflammatory properties, and it is used to treat allergies and cancer in traditional Chinese medicine [13]. Many of the drugs prescribed for psoriasis, dermatitis and skin diseases in China contain paeonol as bioactive compound [14]. The present study investigated the lung cancer-sensitizing potential of paeonol to TRAIL treatment.

EXPERIMENTAL

Cell lines

The H1975 and BGC823 cells were provided by Chinese Academy of Sciences (Shanghai, China). The cells were kept in RPMI-1640 medium containing 10 % FBS and 100 U/mL penicillin and streptomycin.

Cell viability assay

The viability of H1975 and BGC823 cells following paeonol exposure was determined with 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated in 96-well plates in RPMI-1640 medium, each at a density of 0.5×10^6 cells per well and incubated for 24 h. Thereafter, the medium was replaced with fresh medium containing 1, 2, 4 and 8 μM paeonol or TRAIL (25 ng/mL) or paeonol (8 μM) plus TRAIL (25 ng/mL), and cell incubation was continued for 72 h. The 72-h treatment of cells was followed by addition of 20 μL MTT. After incubation for 4 h, the medium was removed and DMSO (150 μL) was added to the plates so as to solubilize the resultant formazan crystals. Then, the absorbance of each well was read at 490 nm in a microplate reader.

Annexin V-FITC/PI double staining for detection of apoptosis

Apoptosis of H1975 and BGC823 cells was analysed with a flow cytometer using Annexin V-FITC/PI kit. The cells were incubated for 72 h in medium containing 8 μM paeonol or TRAIL (25 ng/mL) or paeonol (8 μM) plus TRAIL (25 ng/mL). Cell harvesting was followed by PBS washing (two times) and adjustment of the cells to a density of 2×10^6 cells per ml in 1X binding buffer. Then, the cells were subjected to staining for 20 min with Annexin V-FITC and PI solution in the dark. Flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) was used for examination of apoptotic cells.

Determination of effect of paeonol on expressions of DR4 and DR5

The expressions of DR4 and DR5 in H1975 and BGC823 cells after paeonol treatment were assayed using flow cytometry [15]. The cells were treated with 8 μM paeonol for 72 h and then incubated for 50 min at 4 °C. The antibodies used were monoclonal mouse anti-bodies against DR4 and DR5.

Reverse transcription polymerase chain reaction (RT-PCR) assay

The cells were incubated for 72 h with 8 μM paeonol, or with DMSO as control. Total RNA was extracted from H1975 and BGC823 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Then, 1-μg RNA samples were used to synthesize of cDNA for 20 min at 37˚C employing Primescript RT kit. Roche LightCycler®96 RT-PCR system and SYBR

Premix EX Taq Ⅱ were used for RT-PCR assay. The mixture (20 µL) used for PCR reaction consisted of SYBR Premix EX Taq II (10 µL), forward primer (0.8 µL), reverse primer (0.8 µL), cDNA (2 µL) and sterilized water (6.4 µL). The mixture was amplified under the following conditions: degeneration for 2 min at 93 ˚C, then 40 cycles of denaturation for 5 s at 93 ˚C, followed by annealing/extension for 15 s at 58 ˚C. The $2^{-\Delta\Delta Cq}$ method was used for determination of relative mRNA expression levels [16].

Western blotting

The cells were incubated for 72 h with 8 µM paeonol or DMSO as control and then lysed on ice for 45 min by incubation with lysis buffer. The buffer consisted of sodium chloride (5 M), Nonidet P-40 (10 %), $NaVO_4$ (0.2 M), EGTA (0.1 M), EDTA (0.5 M), phenylmethylsulfonyl fluoride (0.1 M), NaF (1 M), HEPES (1 M), aprotinin (2 µg/mL) and leupeptin (2 µg/mL). The lysate was centrifuged to obtain supernatant and BCA assay kit was used to determine the protein content. The proteins were subjected to SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membranes which were blocked on incubation with 5 % non-fat milk. Then, the membranes were incubated at 4˚C overnight with primary antibodies against JNK, ERK1/2, p38, Bcl-xL, Bcl-2, survivin, Bax, cFLIP, CHOP, xIAP, caspase-3, caspase-8, caspase-9, DcR1, DcR2, DR4 and DR5 (Cell Signaling Technology, Danvers, MA, USA). Thereafter, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. Band visualization was made using ECL detection system (Pierce; Thermo Fisher Scientific, Inc.).

Measurement of ROS

The H1975 and BGC823 cells were incubated for 72 h with 8 µM paeonol or with DMSO (control), followed by incubation at 37°C for 20 min with DCF-DA (20 µM). The level of ROS generated was analysed flow cytometrically at 530 nm.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). One-way ANOVA and Student's t-test were used for statistical analyses which were carried out with GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were taken as statistically significant at $p < 0.05$.

RESULTS

Paeonol and TRAIL inhibited the viability of lung cancer cells

As shown in Figure 1, paeonol at doses of 1, 2, 4 and 8 µM significantly reduced the proliferation of H1975 and BGC823 cells at 72 h, relative to control (($p < 0.05$). In H1975 cells, viability was reduced to 84, 69, 49 and 37 %, respectively, on treatment with paeonol at doses of 1, 2, 4 and 8 µM, respectively, relative to 100 % in control. Treatment with 1, 2, 4 and 8 µM paeonol suppressed BGC823 cell viability to 89, 71, 53 and 39 %, respectively, relative to 99 % in control. However, combination treatment with paeonol (8 µM) and TRAIL (25 ng/mL) resulted in suppression of the proliferation of H1975 and BGC823 cells to 21 and 24 %, respectively.

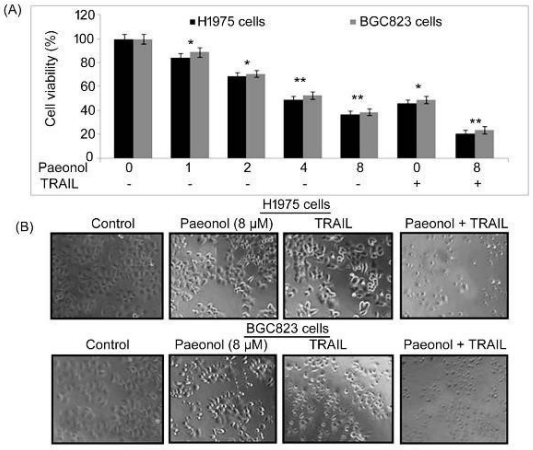

Figure 1: Inhibitory effect of paeonol on viability of H1975 and BGC823 cells. (A) Paeonol treatment for 72 h was followed by determination of viability using MTT assay. **P* < 0.05; ***p* < 0.02, vs. H1975 control and BGC823 control cells, respectively. (B) Images of cells treated with 8 µM paeonol, TRAIL and paeonol plus TRAIL, as seen under a microscope (x200). MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide

Paeonol promoted TRAIL-induced apoptosis of lung cancer cells

Following treatment of H1975 and BGC823 cells with paeonol (8 µM), TRAIL (25 ng/mL) and combination of paeonol and TRAIL, cell apoptosis was analyzed using Annexin V-FITC and PI staining assay. As shown in Figure 2, 8µM paeonol treatment induced apoptosis in H1975 and BGC823 cells to levels of 59.27 and 53.42 %, respectively. Ttreatment with TRAIL induced apoptosis in 32.81 and 31.76 % of H1975 and BGC823 cells, respectively.

However, treatment with combination of paeonol and TRAIL increased apoptosis of H1975 and BGC823 cells to 88.43 and 87.21 %, respectively.

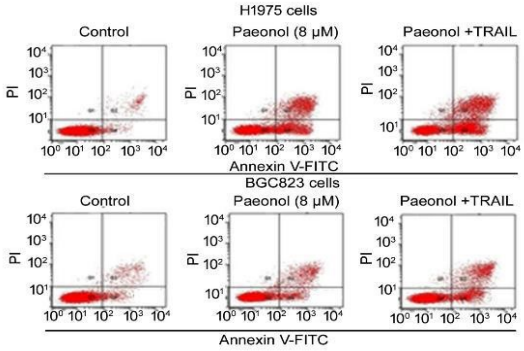

Figure 2: Apoptotic effect of paeonol and TRAIL on lung cancer cells. H1975 and BGC823 cells exposed for 72 h to paeonol (8 μM), TRAIL (25 ng/mL) and combination of paeonol and TRAIL were analysed for apoptotic changes using Annexin V-FITC and PI staining; *$p < 0.05$; **$p < 0.02$, vs. H1975 control and BGC823 control, respectively. TRAIL: tumour necrosis factor-related apoptosis-inducing ligand

Paeonol up-regulated the expressions of DR4 and DR5 and suppressed the expression of decoy receptor

In H1975 and BGC823 cells, the mRNA levels of DR4 and DR5 were significantly increased by treatment with 8 μM paeonol treatment, relative to control ($p < 0.05$; Figure 3 A). Moreover, paeonol markedly enhanced the protein levels of DR4 and DR5 in H1975 and BGC823 cells (Figure 3 B). In contrast, the levels of DcR1 and DcR2 were suppressed in H1975 and BGC823 cells on treatment with 8 μM paeonol (Figure 3 C).

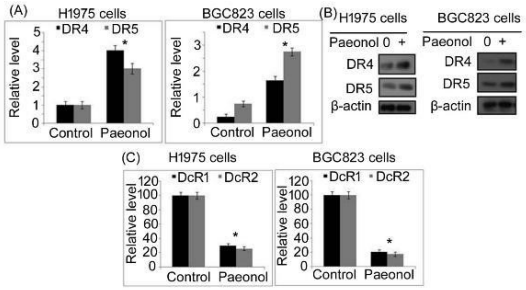

Figure 3: Effect of paeonol on levels of DR4, DR5, DcR1 and DcR2. Levels of DR4 and DR5 in H1975 and BGC823 cells after treatment with 8 μM paeonol, as assayed using (A) RT-PCR, and (B) Western blotting. (C) Levels of DcR1 and DcR2 win H1975 and BGC823 cells at 72 h of treatment with paeonol; *$p < 0.05$ vs. control cells for H1975 and BGC823

Paeonol up-regulated CHOP expression in lung cancer cells

Western blotting of H1975 and BGC823 cells at 72 h of treatment with 8 μM paeonol showed markedly higher CHOP protein expression, relative to the control (Figure 4 A). Moreover, RT-PCR data confirmed that paeonol treatment significantly ($p < 0.05$) raised the mRNA expression of CHOP in H1975 and BGC823 cells (Figure 4 B).

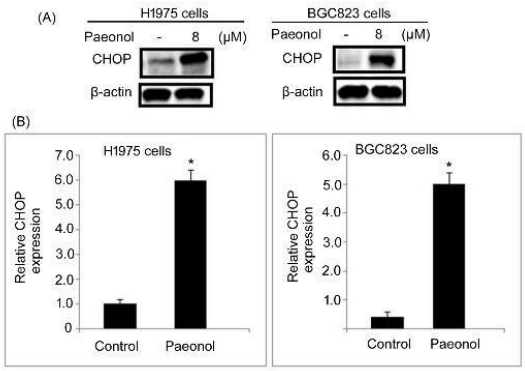

Figure 4: Effect of paeonol on CHOP expressions in H1975 and BGC823 cells. Paeonol-treated H1975 and BGC823 cells were subjected to assay of CHOP expression at 72 h. (A) CHOP protein and (B) CHOP mRNA levels, as assayed using western blotting and RT-PCR assays, respectively; *$p < 0.05$ vs. control cells for H1975 and BGC823

Paeonol suppressed expression of survivin protein and up-regulated expression of pro-apoptotic protein

The concentrations of survivin, Bcl-2, cFLIP and Bcl-xL were elevated in H1975 and BGC823 cells (Figure 5). However, paeonol significantly decreased the protein levels of survivin, Bcl-2, cFLIP and Bcl-xL in H1975 and BGC823 cells, while it markedly up-regulated the protein levels of caspase-3, caspase-8 and caspase-9, relative to the control (Figure 5). In paeonol-treated H1975 and BGC823 cells, Bax level was also markedly elevated, relative to the control cells.

Paeonol induced ROS generation in lung cancer cells

Changes in ROS levels in H1975 and BGC823 cells were measured at 72 h of treatment with 8 μM paeonol (Figure 6). The concentrations of ROS in H1975 and BGC823 cells were significantly elevated, when compared to control, on treatment with paeonol.

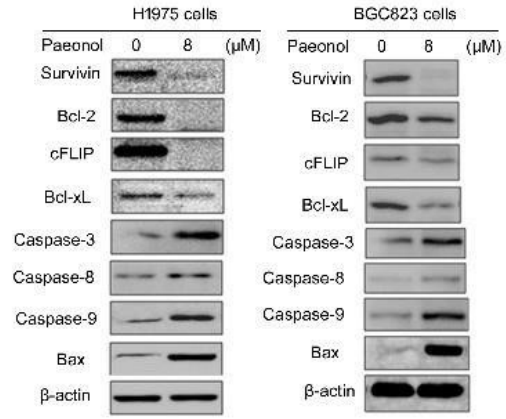

Figure 5: Effect of paeonol on survivin and pro-apoptotic proteins. Following 72 h treatment with 8 μM paeonol, survivin and pro-apoptotic protein levels were determined in H1975 and BGC823 cells using western blot assay

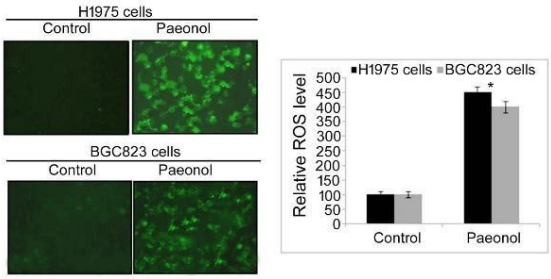

Figure 6: Effect of paeonol on ROS production in lung cancer cells. The H1975 and BGC823 cells were treated with 8 μM paeonol or DMSO (control) for 72 h, followed by determination of ROS levels using flow cytometry

MAPKs mediated tangeretin-induced upregulation of TRAIL receptors

Treatment with paeonol markedly elevated p-ERK and p-JNK in H1975 and BGC823 cells, relative to the control cells (Figure 7). The increases in p-ERK level in H1975 and BGC823 cells on treatment with paeonol were much higher those of p-JNK. However, no significant increases in p-p38 expression were observed in H1975 and BGC823 cells on exposure to paeonol.

DISCUSSION

Although tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been found to be effective in cancer therapy, its anti-tumor potential is limited because of the development of resistance by tumor cells [7,8]. Studies have revealed lower levels of death receptors and higher expressions of Bcl-2, survivin and cFLIP in TRAIL-resistant tumor cells [8,9,15,16].

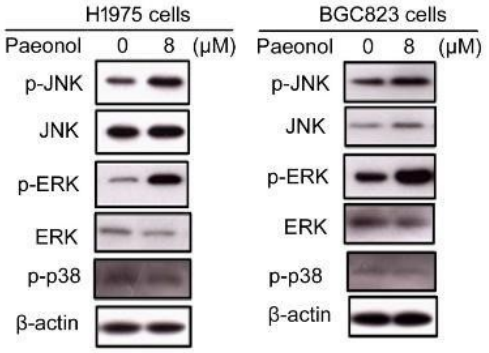

Figure 7: Effect of paeonol on activation of JNK/ERK1/2/p38. JNK/ERK1/2/p38 activation in H1975 and BGC823 cells was determined at 72 h of paeonol treatment, using western blotting

The anti-apoptotic protein xIAP is also over-expressed in tumor cells resistant to TRAIL [16]. Therefore, sensitization of tumor cells to TRAIL promotes apoptosis, with tremendous potential for treatment of tumors.

Some molecules sensitize carcinoma cells to TRAIL, thereby efficiently enhancing apoptosis [11,17]. It has been reported that xIAP inhibits caspase-3, caspase-7 and caspase-9, whereas cFLIP competes for Fas-associated death domain in TRAIL-resistant tumor cells [9,16]. In the present study, paeonol exhibited cytotoxic effect on H1975 and BGC823 cells and significantly suppressed their proliferation. Interestingly, the cytotoxic effect produced by combined treatment with paeonol and TRAIL was much higher than that of paeonol or TRAIL when used separately. This provides evidence that paeonol sensitized H1975 and BGC823 cells to the cytotoxic effects of TRAIL.

Findings from MTT assay were consistent with results obtained using Annexin V-FITC and PI assay. Although apoptosis was induced significantly in H1975 and BGC823 cells on treatment with paeonol or TRAIL, the strongest effect was produced by paeonol/TRAIL combination. Moreover, paeonol targeted expressions of various proteins related to survival of cells i.e., Bcl-2, survivin, cFLIP and xIAP in H1975 and BGC823 cells. Therefore, paeonol sensitized H1975 and BGC823 cells to TRAIL by suppressing the protein expression of survivin. TRAIL-mediated apoptosis in carcinoma cells is enhanced by some compounds via enhancement of the activities of caspases [11,18].

In general, TRAIL binds to DR4 and DR5 receptors and subsequently activates executor

caspases thereby inducing apoptosis of carcinoma cells [22]. Death receptors are down-regulated in TRAIL-resistant cells, resulting in enhancement of DR4 and DR5, and promotion of apoptosis [9,18,19]. Over-expressions of DcR1 and DcR2 have been reported in TRAIL-resistant cells, resulting in resistance to apoptosis [20]. The transcriptional factor, CHOP, on binding to the promoter region of DR5 receptor, up-regulates its expression [21].

In the present study, paeonol enhanced the expressions of DR4 and DR5 in H1975 and BGC823 cells, while it downregulated the expressions of DcR1 and DcR2. In addition, there were marked increases in CHOP level in H1975 and BGC823 cells on treatment with paeonol. Death receptors are induced in carcinoma cells by enhanced production of reactive oxygen species [17]. Moreover, the level of stress-regulated protein, CHOP is also enhanced by over-production of ROS [21].

Studies have shown that TRAIL-mediated increase in DR5 expression and induction of apoptosis by chemotherapeutic agents occur via ROS over-production [11,22]. During TRAIL-induced apoptosis, the levels p-ERK, p-JNK and p-p38 levels are markedly increased via ROS signalling pathway. In the present study, paeonol treatment promoted ROS levels and activated ERK and JNK in H1975 and BGC823 cells. Thus, paeonol sensitized H1975 and BGC823 cells to TRAIL through ROS-mediated activation of the JNK/ERK pathway.

CONCLUSION

Paeonol inhibits the viability of lung cancer cells via upregulation of death receptors, activation of JNK/ERK-CHOP pathway and generation of ROS. Therefore, paeonol has promising potential as an agent for lung cancer treatment.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. All the experiments were performed by Yanqing Fan and Xiaoyan Chen under the supervision of Guizhi Zhang. Xiaoyan Chen collected the data and did statistical analysis. Yanqing Fan did most part of experiments.

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