Original Article Growth inhibition and apoptosis induction of pPeOp protein in human colon cancer cell line SW620

Zhongxia Lu^{1*}, Wenjun Xu^{1*}, Meiai Lin², Xiaofang Zhou³, Yitao Chen¹

¹College of Life Sciences, ²The Second Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou 310053, China; ³Zhejiang Cancer Hospital, Hangzhou 310022, Zhejiang, China: ^{*}Equal contributors.

Received January 27, 2017; Accepted March 22, 2018; Epub June 15, 2018; Published June 30, 2018

Abstract: The pPeOp is a protein extracted from the sclerotium of *Omphalia lapidescens*. Herein, we explored the effect of pPeOp inhibition of cell proliferation and induction of cell apoptosis in colon cancer cell line SW620. Cell viability was detected by the MTS assay. Cell proliferation was tested by the EdU assay. Apoptosis was assessed by flow cytometry and Hoechst staining. The apoptosis proteins expression of Fas, FasL, Cleaved-caspase-3, Caspase-8, and Bcl-2 was analyzed by western blotting. We treated SW620 colon cancer cells with 0, 30, 60, 90 or 120 µg/ml pPeOp and studied the impact it had on the viability, the proliferation and apoptosis of treated cells. A 24-hour treatment with pPeOp inhibited the viability of SW620 cell in a dose-dependent manner, wherein 85.24 µg/ml was sufficient to inhibit cell viability by 50% (IC50). Besides, the proliferation of SW620 cells was significantly inhibited by pPeOp. Our microscopy images show that SW620 cells treated with > 60 µg/ml pPeOp became round in morphology and appeared to shrink. pPeOp also enhanced the rate of apoptosis in a dose-dependent manner. Fas, FasL, Cleaved-caspase-3, and Caspase-8 were significantly up-regulated and Bcl-2 was significantly down-regulated relative to control cells. Our data suggest that pPeOp is able to suppress tumor growth by inducing the apoptosis of human colon cancer cells. Accordingly, pPeOp may serve as a novel therapeutic agent for colorectal cancer.

Keywords: Apoptosis, colorectal cancer, Fas/FasL, Omphalia lapidescens, pPeOp

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth most common cause of cancer deaths worldwide, wherein 608,000 patients succumb to the disease each year [1]. Although considerable advances in screening and treatment have been made, a significant number of patients are still diagnosed during the late stages of the disease associated with dismal outcomes [2].

Omphalia lapidescens - "Lei Wan" in Chinese is a fungus used in Traditional Chinese Medicine (TCM) to treat numerous parasites, including roundworms [3]. This fungus was originally reported in the *Shen nong Materia Medica* and was attributed to the stomach and large intestine meridians, which indicated its potential effects on these two organs [4]. Numerous studies have reported that *O. lapidescens* possesses anti-tumor properties, including suppressing proliferation and inducing apoptosis of cancer cells [5, 6]. Accordingly, the China Food and Drug Administration (CFDA) have approved Lei Wan pill and Lei Wan capsule as anti-tumor drugs.

pPeOp is a protein extracted from the sclerotium of *O. lapidescens* with PVP extraction buffer. Previous studies demonstrated that pPeOp promoted the apoptosis, inhibited migration and arrested cell cycle of MC-4 gastric cancer cells, whilst causing no toxicity to MC-1 normal gastric cells [3, 4].

To date, however, no studies have investigated its efficacy for treating colorectal cancer. The present study aimed to investigate the influence that pPeOp has on the cell viability and apoptosis of SW620 human colon cancer cells. Besides, cells treated with 120 ug/ml PVP was regarded as a negative control. Furthermore, we also studied the expression of apoptosisassociated proteins.



Figure 1. Morphology analysis and MTS cell viability assay. Following treatment with 0-120 μ g/ml pPeOp or 120 μ g/ml PVP for 24 h, cell morphology was observed under the light microscope. A: Control SW620 cells. B: PVP treated SW620 cells. C-F: SW620 cells treated with different doses of pPeOp. G: MTS assay cell viability of SW620 cells (**P* < 0.05, ***P* < 0.01, compared with the control group).

Materials and methods

pPeOp extraction and purification

The fruiting body of *O. lapidescens* was provided by Fang Hui Chun Tang (Hangzhou, China). Protein extraction and purification were performed according to a previously published protocol [4].

Cell line and cell culture

Human SW620 colon cancer cells were provided by the Zhejiang Provincial Center for Disease Control and Prevention (ZJCDC). SW620 cells were cultured in RPMI 1640 medium (Hangzhou Genom Biomedical Company) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂.

MTS assay

Cell viability was determined by MTS assay. SW620 cells $(1 \times 10^5/\text{ml})$ were seeded in 96-well plates and cultured with varying concentration pPeOp (0, 30, 60, 90 or 120 µg/ml) for 24 h at 37°C. Subsequently, 20 µl MTS (Promega Corporation) were added to each well, agitated lightly for 30 s, and then incubated for 2 h at 37°C in the dark. The optical density (OD) was measured at 490 nm using a microplate reader (Labsystems).

5-ethyny-2'-deoxyuridine (EdU) cell proliferation assay

EdU assay was used to test cell proliferation. Human SW620 cells (1 × 10⁵/ml) were seeded in 24-well plates and cultured with different dose of pPeOp (0, 30, 60, 90 or 120 µg/ml) for 24 h. BeyoClick[™] EdU Cell Proliferation Assay Kit with Alexa Fluor 594 was purchased from Beyotime Biotechnology. EdU assay were followed by product instruction. Briefly, EdU solution (10 µM, dissolve in culture medium) were employed to cultured SW620 cells for 2 h at room temperature. Then, 4% Paraformaldehyde (1 ml/well) was added and incubated for 30 min in the room temperature. After wash three times with wash buffer (Beyotime), cells were incubated with 0.3% Triton X-100 (1 ml/well) for 15 min at the room temperature. Then, cells were incubated with Click Reaction solution (Beyotime) for 30 min in the dark. After wash three times with wash buffer, the Hoechest 33342 solution was applied to incubate cells 30 min in the dark. Stained cells were examined using a Leica DMIRE inverted fluorescence microscope system (Leica Microsystems Corp). Under the fluorescence microscope, five

pPeOp induces SW620 cells apoptosis



fields were randomly selected to calculate the number of EdU-positive cells.

Flow cytometry assay

Flow cytometry assay was employed to detect apoptosis and necrotic cells by Annexin V-FITC and propidium iodide (PI) double staining. After treated with gradient concentration pPeOp (0, 30, 60, 90 or 120 μ g/ml) for 24 h, SW620 cells were subsequently collected and re-suspended with ice-cold PBS. Next, cells were stained with the FITC/Annexin V/PI reagent (BD Biosciences) and incubated for 15 min at room temperature in the dark. Apoptotic cells were analyzed using a Beckman FC500 flow cytometer (Beckman Coulter).

Hoechst staining assay

Human SW620 cells (1 \times 10⁶ cells/well) were seeded in 6-well plates and then treated with

pPeOp (0, 30, 60, 90 or 120 μ g/ml) or PVP (120 μ g/ml) for 24 h. Treated cells were stained with the Hoechst 33258 reagent (Beyotime) of the apoptotic nuclear chromatin staining kit. Stained cells were examined for signs of apoptosis, as determined by condensed and fragmented nuclei, and imaged using a Leica DMIRE inverted fluorescence microscope system (Leica Microsystems Corp).

Western blotting assay

Treated cells were harvested and total protein was extracted with ice-cold lysis buffer (Beyotime) and quantitated with a BCA protein assay kit (Keygen Biotech). Next, 20 μ g protein was resolved by SDS-PAGE, transferred onto a PVDF membrane (Millipore Company) and blocked with skim milk solution for 2 h. The membrane was then incubated with rabbit antibodies against Fas, FasL, Cleaved-caspase-3, Caspase-8, and Bcl-2 overnight at 4°C. All pri-



Figure 3. Morphological analysis of SW620 cells by Hoechst staining. After treated with 0-120 µg/ml pPeOp or 120 µg/ml PVP for 24 h, cell nuclei fragments were observed under a fluorescence microscopy. A: Control SW620 cells. B: PVP treated SW620 cells. C-F: SW620 cells treated with different doses of pPeOp (30, 60, 90 or 120 µg/ml).

mary antibodies were purchased from Cell Signaling Technology. Membranes were washed three times with TBST and incubated with a goat anti-rabbit secondary antibody (Sangon) for 2 h and washed three times with TBST. Blots were visualized by an Aplegen Omega Lum G Gel Documentation System.

Statistical analysis

All experiments were conducted in triplicate. Data are expressed as the mean ± SD. SPSS software was used for statistical analyses. Results of the MTS cell viability assay and Flow cytometry-based analysis were subjected to one-way analysis of variance (ANOVA). Western blotting results were quantitated using Image J software and analyzed by ANOVA.

Results

pPeOp changes the morphology of SW620 cells

Light microscopy was applied to analyze the morphological changes of the SW620 cells. Control cells were used to determine baseline

cellular morphology (**Figure 1A**). When treated with PVP buffer ($120 \mu g/ml$), as shown in **Figure 1B**, SW620 cells retained a normal morphology compared to control cells. When exposed to $30-120 \mu g/ml$, however, cells became markedly damaged, reverting to a spherical morphology and showing signs of shrinkage (**Figure 1C-F**).

Under a fluorescence microscopy, morphological changes of cell nuclei were further observed after Hoechst 33258 staining. In control group, SW620 cells nuclei remain round in shape and were homogeneously stained by Hoechst 33258 (**Figure 3A**). A similar morphology was observed in the PVP-treated group (**Figure 3B**). However, as shown in **Figure 3C-F**, some fragmentation and condensation of nuclei were seen. Taken together, these results show that pPeOp induced morphological changes in a dose-dependent manner; changes that were not observed when cells were not exposed to pPeOp.

pPeOp inhibiting cell viability of SW620 cells

The MTS assay was employed to measure the inhibitory activity of pPeOp on cell viability.



Figure 4. Cell apoptosis was detected by flow cytometry analysis. The proportion of SW620 cells treated with 0-120 μ g/ml pPeOp or 120 μ g/ml PVP for 24 h undergoing apoptosis. A: Control SW620 cells. B: PVP treated SW620 cells. C-F: SW620 cells treated with different doses of pPeOp (30, 60, 90 or 120 μ g/ml). G: Apoptosis rate of cells with different treatments. H: Survival rate of cells with different treatments (**P* < 0.05, ***P* < 0.01, compared with the control group).



Figure 5. pPeOp alters the expression of apoptosis regulatory proteins by Western blotting analysis. SW620 cells treated with 0-120 µg/ml pPeOp or 120 µg/ml PVP for 24 h. Expression of apoptosis-related proteins (Fas, FasL, Cleaved-caspase-3, Caspase-8, and Bcl-2) as shown by western blotting. (**P* < 0.05, ***P* < 0.01, compared with the control group).

SW620 cells were exposed to varying concentration of pPeOp or 120 µg/ml for 24 h, after which the cells were assayed for their viability. As shown in **Figure 1G**, when compared with control group, the inhibition of cell viability in colon cancer cells become more significant with increasing concentration of pPeOp (P < 0.05 and P < 0.01). On the contrary, there was no significant difference in the viability of cells treated with PVP. The concentration at which SW620 viability was inhibited by 50% (IC50) was 85.24 µg/ml. These data indicate that pPeOp inhibited the viability of SW620 cancer cells in a dose-dependent manner.

pPeOp(µg/ml)

pPeOp exhibits anti-proliferative activity against SW620 cells

The EdU cell proliferation test was used to assay cell proliferation. As shown in **Figure 2**, the relative EdU-positive cell numbers significantly decrease with increasing concentration of pPeOp (P < 0.05 and P < 0.01). On the other hand, there was no significant difference in the EdU-positive cell numbers with PVP. Our data suggested the proliferation activity of pPeOp was significantly inhibited by pPeOp.

pPeOp triggers apoptosis in SW620 cells

To test the effect of pPeOp on apoptosis of colon cancer cells, SW620 cells were treated

with varying concentration of pPeOp, and then stained with Annexin V-FITC and PI, and the apoptotic cells were distinguished by flow cytometry. Flow cytometry-based analysis indicated that the percentage of SW620 cells undergoing apoptosis was significantly increased (**Figure 4A-F**). Furthermore, the number of surviving cells was significantly decreased with the increasing dose of pPeOp (**Figure 4H**), which is consistent with the cell viability obtained by MTS analysis. Thus, our data confirm that pPeOp is able to induce apoptotic in a dose-dependent manner.

pPeOp alters the expression of apoptosis regulatory proteins

To elucidate the mechanism underlying the pPeOp-induced apoptosis of SW620 cells, we assayed the expression of Fas, FasL, Cleaved-caspase-3, Caspase-8 and Bcl-2 by Western blotting. Following 24 h of treatment with 30-120 pPeOp µg/ml, as shown in **Figure 5**, the expression of Fas, FasL, Cleaved-caspase-3 and Caspase-8 were significantly elevated (P < 0.05, P < 0.01), whereas the expression of Bcl-2 was significantly down-regulated (P < 0.05, P < 0.01). By contrast, as shown in **Figure 5**, there was no significant difference between cells treated with 120 µg/ml PVP and control cells (P > 0.05). Accordingly, the expression of numerous apoptosis regulators

is modulated post pPeOp treatment. These results show that pPeOp modulates the expression of apoptosis-related proteins in colon cancer cells.

Discussion

Apoptosis is a normal physiological process essential to development and homeostasis [7], by which organisms eliminate superfluous, harmful or unhealthy cells [8, 9]. Cells undergoing apoptosis go through a series of characteristic morphological changes, including cell shrinkage, membrane blebbing, nuclear fragmentation and poly-nuclear DNA fragmentation [10].

Apoptosis is activated mainly through two distinct pathways-namely the death receptor (extrinsic) pathway or mitochondrial (intrinsic) pathway [11, 12]. The death receptor pathway is the most detailed and well-studied apoptosis pathway and the one in which the Fas/FasL and TNFR pathways transduce through. Fas is a type I-membrane protein and an important member of the tumor necrosis factor receptor (TNFR) superfamily [13]. When the Fas ligand (FasL) binds to Fas, a death-inducing signaling complex (DISC) is formatted, which includes the adaptor recruitment protein FADD and Caspase-8. Then, activation of Caspase-8 initiates a caspase cascade leading to the activation of Caspase-3, which results in the execution of apoptosis [14]. The Bcl-2 family of proteins is located in the inner mitochondrial membrane wherein they play a pivotal role in mitochondrial apoptosis pathway [15]. The Bcl-2 family proteins subdivided into three classes: anti-apoptosis regulators (such as Bcl-2, Bcl-xl, Bcl-w), pro-apoptotic regulators (such as Bax, Bak) and the BH3 (Bcl-2 Homology 3) only proteins [16]. In the mitochondrial apoptosis pathway, the apoptosis is initiated by Bak and Bax through the process of increasing the mitochondrial outer member permeability and release the cytochrome c to the cytosol. However, Bcl-2 protein inhibits this process [17]. Numerous proteins and enzymes are involved in the initiation, amplification, or even suppression, of apoptosis, but it is generally the cysteine and aspartic acid-containing proteases (Caspases) that mediate the final process of cell death [18, 19].

In the present study, SW620 cells treated with different doses of pPeOp turned round and appeared to shrink. Besides, we subsequently showed by flow-cytometry that pPeOp induced apoptosis in a dose-dependent manner. For the death receptor apoptosis pathway, the initiation proteins (Fas, FasL) were significantly up-regulated. Caspase-8, act as amplification proteins in death receptor apoptosis pathway. was also significantly up-regulated. Besides, cells treated with pPeOp significantly up-regulated the execution protein (Cleaved-caspase-3). Furthermore, the protein levels of Bcl-2 significantly down-regulated. Therefore, pPeOpinduced apoptosis in SW620 cells may be related to the death receptor and mitochondrial pathway. In summary, pPeOp significantly inhibits proliferation and induces apoptosis in SW620 cancer cells and has the potential to be used as a treatment for CRC.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation Project (81374023) and the Zhejiang Provincial Natural Science Foundation of China under Grant (No. LY18H280008). The present study was also supported by the Zhejiang Provincial Medical and Health Science and Technology Project (2016KYA033).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yitao Chen, College of Life Sciences, Zhejiang Chinese Medical University, Hangzhou 310053, Zhejiang, China. Tel: +86-18758167896; +86-571-86613624; Fax: +86-571-86613600; E-mail: cytworld@163.com

References

- [1] Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RG, Barzi A and Jemal A. Colorectal cancer statistics, 2017. CA Cancer J Clin 2017; 67: 177-193.
- [2] Shaukat A, Mongin SJ, Geisser MS, Lederle FA, Bond JH, Mandel JS and Church TR. Long-term mortality after screening for colorectal cancer. N Engl J Med 2013; 369: 1106-1114.
- [3] Yang YL, Gong WY, Chen FF, Chen LC and Chen YT. pPeOp from Omphalia lapidescens Schroet induces cell cycle arrest and inhibits the migra-

tion of MC-4 gastric tumor cells. Oncol Lett 2017; 14: 533-540.

- [4] Chen YT, Lu QY, Lin MA, Cheng DQ, Ding ZS and Shan LT. A PVP-extract fungal protein of Omphalia lapideacens and its antitumor activity on human gastric tumors and normal cells. Oncol Rep 2011; 26: 1519-1526.
- [5] Ohno N, Miura T, Saito K, Nishijima M, Miyazaki T and Yadomae T. Physicochemical characteristics and antitumor activities of a highly branched fungal (1----3)-beta-D-glucan, OL-2, isolated from Omphalia lapidescens. Chem Pharm Bull (Tokyo) 1992; 40: 2215-2218.
- [6] Saito K, Nishijima M, Ohno N, Yadomae T and Miyazaki T. Structure and antitumor activity of the less-branched derivatives of an alkali-soluble glucan isolated from Omphalia lapidescens. (Studies on fungal polysaccharide. XXX-VIII). Chem Pharm Bull (Tokyo) 1992; 40: 261-263.
- [7] Chinnaiyan AM, O'Rourke K, Tewari M and Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 1995; 81: 505-512.
- [8] Hay BA, Wassarman DA and Rubin GM. Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell 1995; 83: 1253-1262.
- [9] Wang B, Liu J and Gong Z. Resveratrol induces apoptosis in K562 cells via the regulation of mitochondrial signaling pathways. Int J Clin Exp Med 2015; 8: 16926-16933.
- [10] Degterev A and Yuan J. Expansion and evolution of cell death programmes. Nat Rev Mol Cell Biol 2008; 9: 378-390.

- [11] Green DR. Apoptotic pathways: paper wraps stone blunts scissors. Cell 2000; 102: 1-4.
- [12] Wang X. The expanding role of mitochondria in apoptosis. Genes Dev 2001; 15: 2922-2933.
- [13] Nagata S. Apoptosis by death factor. Cell 1997; 88: 355-365.
- [14] Li H, Xu J, Wang X and Yuan G. Protective effect of ginsenoside Rg1 on lidocaine-induced apoptosis. Mol Med Rep 2014; 9: 395-400.
- [15] Sentman CL, Shutter JR, Hockenbery D, Kanagawa O and Korsmeyer SJ. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 1991; 67: 879-888.
- [16] Martinou JC and Youle RJ. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell 2011; 21: 92-101.
- [17] Ouyang M, Liu H, Yang K, Jiang W, Ding Q, Yu X and Chen W. Olecular mechanism underlying the myeloperoxidase induced apoptosis of HU-VEC-12 cells. Int J Clin Exp Med 2014; 7: 879-885.
- [18] Shalini S, Dorstyn L, Dawar S and Kumar S. Old, new and emerging functions of caspases. Cell Death Differ 2015; 22: 526-539.
- [19] Liu WQ, Kang M, Qin YT, Wei ZX and Wang RS. Apoptosis-inducing effects of cetuximab combined with radiotherapy and hypothermia on human nasopharyngeal carcinoma CNE cells. Int J Clin Exp Med 2015; 8: 2182-2189.