Emodin induces Panc-1 cell apoptosis via declining the mitochondrial membrane potential

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Abstract. In this study, we investigated the apoptotic effect of emodin on human pancreatic cancer cell line Panc-1 *in vitro* and *in vivo* as well as the possible mechanisms involved. *In vitro*, human pancreatic cancer cell line Panc-1 was exposed to varying concentrations of emodin (0, 10, 20, 40 or 80 μmol/l). Then the mitochondrial membrane potential (MMP) was analyzed by JC-1 staining, cell apoptosis was analyzed by flow cytometry (FCM) and cell proliferation was analyzed by MTT. *In vivo*, nude mice orthotopically implanted were randomly divided into five groups to receive treatments by different doses of emodin: control group (normal saline 0.2 ml), E_{10} group (emodin 10 mg/kg), E_{20} group (emodin 20 mg/kg), E_{40} group (emodin 40 mg/kg) and E_{80} group (emodin 80 mg/kg). Each mouse was treated 5 times by intraperitoneal injection of emodin every 3 days. During the treatment, the feeding stuff was recorded. One week after the last treatment, we recorded the body weight and the maximum diameter of tumor in each group before the mice were sacrificed. Then the cell apoptosis of the tumor was tested by TUNEL assay. The results *in vitro* showed that the MMP of the cells declined and the apoptosis rate increased with the emodin concentration increasing and the cell proliferation of each group was inhibited in a dose- and time-dependent manner by emodin. The feeding stuff curve did not decline significantly in E_{40} group and the apoptosis rate of the tumor cells in this group was higher than the lower-dose groups. Taken together, our results demonstrate that emodin may induce the pancreatic cancer cell apoptosis via declining the MMP and a moderate dose of emodin improved the living state of the model mice.

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Introduction

Pancreatic cancer is known for its high mortality rate. It is one of the most common causes of cancer mortality in developed countries (1). Due to the lack of effective-specific diagnosis methods, 26% of patients are in the middle-late stage when being diagnosed and only <10% of patients has a surgery opportunity, and even if the patients have the operation, they still need a series of comprehensive therapies, including chemotherapy (2). In the past decades, gemcitabine was prefered as the first-line drug for pancreatic cancer chemotherapy, but the benefits were very limited, so that new chemotherapy regimens for pancreatic cancer were explored and tested. But new chemotherapy results did not achieve considerable achievement and still could not replace gemcitabine as the gold standard for clinical treatment (3). Thus, it is extremely urgent to look for agents that have low toxicity and high-efficency.

Emodin, 1,3,8-trihydroxyl-6-methyl anthraquinone, is a monomer of Chinese Herb separated from *Rhubarb Genera* and *Polygonum* and *Rhamnus* and *Folium Sennae*. A number of studies show that emodin has a good effect on treating prostate (4), colorectal (5) and pancreatic cancer (6). In this study, we found that the Panc-1 cells' MMP declined and apoptosis rate increased dose-dependently with emodin treatment and the cell proliferation of each group was inhibited in a dose-and time-dependent manner. The feeding stuff curve did not decline significantly in the group of the mice treated with emodin at the dose of 40 mg/kg and the apoptosis rate of the tumor cells was higher than the lower-dose groups. These results demonstrated that emodin could induce Panc-1 cells apoptosis via declining the MMP and a moderate dose of emodin improved the living state of the model mice.

Materials and methods

Chemicals and reagents. Emodin and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Emodin was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO used *in vitro* was <0.1% and that *in vivo* <1%. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA were

obtained from Gibco-BRL (Invitrogen, Grand Island, NY, USA).

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) kit was purchased from Beyotime Biotechnology (Haimen, China). Annexin V-FITC cell apoptosis detection kit was purchased from Nanjing KeyGen Biotechology Co., Ltd., (Nanjing, China). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) kit was purchased from Roche Co. (Mannheim, Germany).

Cell line and cell culture. Human pancreatic cancer cell line Panc-1 was purchased from the American Type Culture Collection and cultured in DMEM with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37˚C under a humidified 5% CO_2 atmosphere. Cells were passaged at 80-90% confluence.

JC-1 analysis. Panc-1 cells were placed in 6-well plates and cultured overnight. Then, Panc-1 cells were cultured in the medium with 0, 10, 20, 40 or 80 μmol/l emodin for 24 h. After that, Panc-1 cells were stained with 5 mg/ml JC-1 for 20 min at 37˚C in the dark. Then MMP depletion was observed under a fluorescence microscope.

FCM analysis. Panc-1 cells ($4x10^5$) were seeded to each 6-well plate. When cells adhered to the culture flask wall, they were treated with 0, 10, 20, 40 or 80 μmol/l emodin for 24 h before collected for trypsin digestion. The cell apoptosis were then detected according to the instructions of Annexin V-FITC cell apoptosis detection kit and assessed by FCM (Becton-Dickinson, San Jose, CA, USA).

MTT analysis. Panc-1 cells were cultured in the medium with various concentrations of emodin (0, 10, 20, 40 or 80 μmol/l) for different time (12, 24, 48 or 72 h) and then the viability of Panc-1 cells was determined by MTT. Briefly, cells were plated at a density of $5x10^3$ cells/well in 96-well microtiter plates. After treatment, 20 μl of MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and the plates were incubated. The supernatant was aspirated and the MTT formazan was dissolved in 150 μl of DMSO. The plates were mixed for 10 min on a gyratory shaker and absorbance was measured with an ELISA reader (Bio-Tek ELx800, Winooski, VT, USA) at a wavelength of 490 nm. Experiment was repeated thrice. Inhibition rate (%) = [1 - (dosing absorbance - blank absorbance)/(control absorbance - blank absorbance)] x 100%.

Experimental animals. Male nude mice [4-5 weeks old, BALB/c (nu/nu)), weight 17-18 g) were purchased from Shanghai Cancer Institute for Tumor Implantation and maintained in a specific pathogen free (SPF) environment in the Animal Experiment Center of the Wenzhou Medical College. All animal studies were approved by the Animal Research and Ethics Committee of the Wenzhou Medical College.

Model establishment and experiment scheme. Suspensions consisting of Panc-1 cells in serum-free medium, with >90% viability, were used for model establishment. Mice were anesthetized with 2% pentobarbital sodium solution and a small left abdominal flank incision was made. Panc-1 cells ($5x10^6$) in 100 μl serum-free medium were injected into the subcapsular region of the pancreas using a 27-gauge needle. The abdominal wound was closed in one layer.

After three weeks, the model mice were divided into five groups randomly to receive different treatment: control group (N, physiological saline), E_{10} group (emodin, 10 mg/kg), E_{20} group (emodin, 20 mg/kg), E_{40} group (emodin, 40 mg/kg), E_{80} group (emodin, 80 mg/kg). Each mouse was treated 5 times by intraperitoneal injection of emodin every 3 days. The feeding stuff intake was recorded before every treatment. One week after the last treatment, the body weight was measured and the mice were euthanized with 2% sodium pentobarbital, followed by measuring the largest diameter of tumors. Finally, implanted tumors were formalin-fixed and paraffin-embedded for subsequent TUNEL assay.

TUNEL analysis. We assessed the degree of tumor apoptosis with the TUNEL assay after the nude mice were sacrificed. TUNEL staining of paraffin-embedded tumor sections was done with the TUNEL kit according to the instructions. Laser scanning confocal microscope (Olympus BX51, Japan) under 400-fold observation camera was used, with excitation wavelength 488 nm and emission wavelength 568 nm. We observed 10 field visions of the strongest fluorescence on each slice.

Statistical analysis. SPSS 17.0 was used for statistical analysis. Data are presented as the means ± SD. Differences among groups of cells or mice were analyzed by one-way ANOVA followed by unpaired Student's t-test. $P<0.05$ was considered statistically significant.

Results

Emodin induces MMP decline in human pancreatic cancer cell line Panc-1. JC-1 fluorescent dyes can gather in the matrix of mitochondria and produce red fluorescence. If the MMP is reduced, JC-1 can not gather the matrix so that JC-1 exists in the matrix as a monomer, producing green fluorescence. JC-1 fluorescent color changed from red to green along with the increasing concentration of emodin, suggesting that MMP declined along with the increasing concentration of emodin (Fig. 1).

Emodin induces apoptosis of human pancreatic cancer Panc-1 cells. We employed Annexin V-FITC cell apoptosis detection kit to detect cell apoptosis. Our study showed that cell apoptosis rate of each group was upregulated dose-dependently by emodin (Fig. 2).

Emodin inhibits the proliferation of human pancreatic cancer cell Panc-1. The cell proliferation-inhibition rate was detected by MTT assay. We found that cell proliferation was inhibited dose- and time-dependently. With treatment of 40 μmol/l emodin, the inhibition rate of each time point (12, 24, 48 or 72 h) is 3.66±0.99%, 17.67±0.49%, 38.13±0.11% and 54.73±0.83%, respectively (Fig. 3).

Emodin increases the amount of feed of model mice. We recorded the feeding stuff eaten by each mouse each day. Data

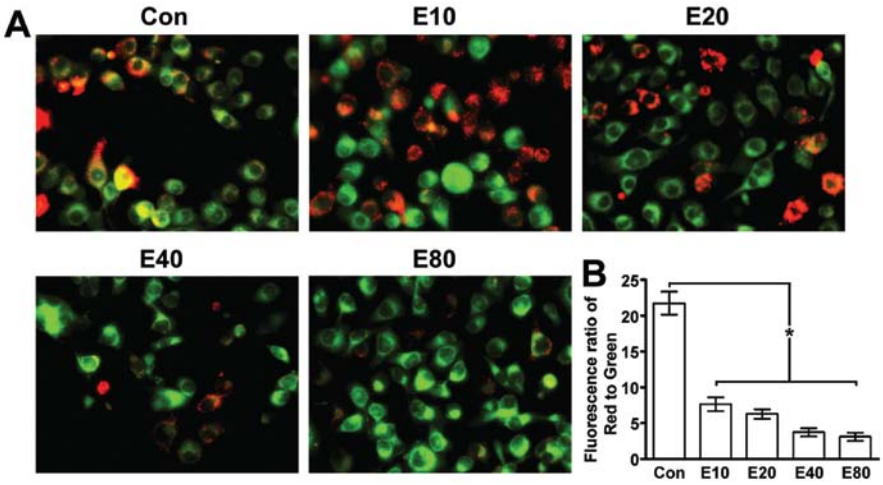

Figure 1. Effect of various concentrations of emodin on the MMP in Panc-1 cells. (A) Representative pictures are shown. JC-1 fluorescent dyes can gather in the matrix of mitochondria and produce red fluorescence. As the MMP declined, JC-1 can not gather the matrix so that JC-1 exist in the matrix as monomer, producing green fluorescence. (B) The ratio of the red (OD1) and green (OD2) optical density is shown. As the concentration of emodin increased, the ratio declined obviously. *P<0.05, compared to the control group.

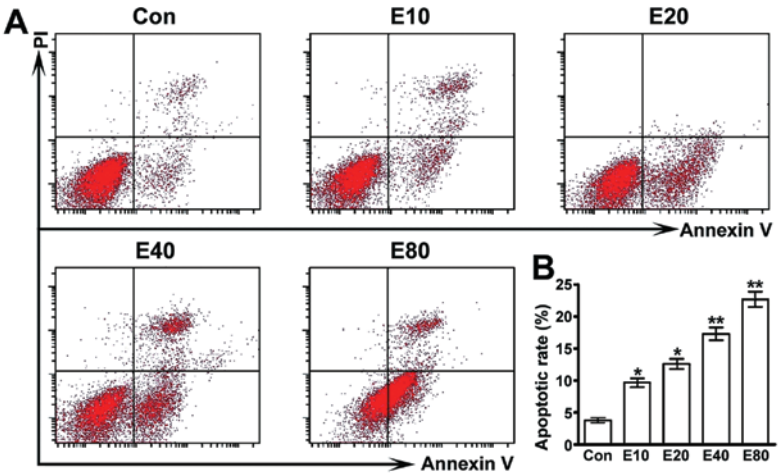

Figure 2. Effect of various concentrations of emodin on the apoptosis of Panc-1 cells. (A) Representative dot-plots illustrating the apoptotic status of Panc-1 cells. (B) The percentage of apoptotic Panc-1 cells. *P<0.05, compared to the control group; **P<0.01, compared to the control group.

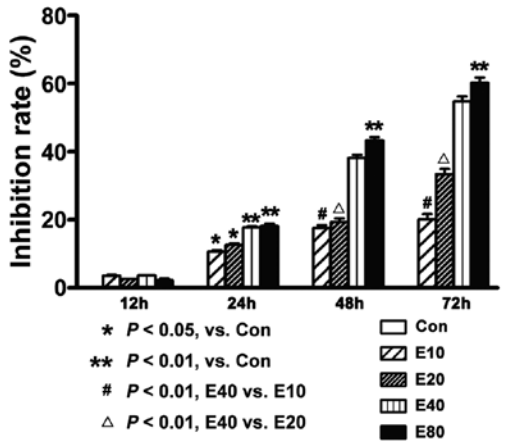

Figure 3. Effect of various concentrations of emodin on the proliferation of Panc-1 cells *in vitro*. The cells without drug treatment were used as the control. Emodin inhibited the proliferation of Panc-1 cells in a dose- and time-dependent manner. Data are expressed as the means ± SD. *P<0.05, compared to the control group; **P<0.01, compared to the control group; #P<0.01 and ΔP<0.01, compared to 40 μmol/l emodin treated cells.

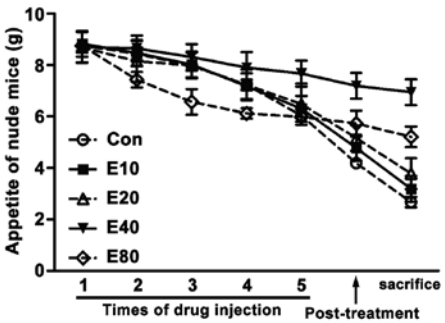

Figure 4. Effect of various concentrations of emodin on the feeding stuff eaten by nude mice. Feeding stuff that the mice of control group ate declined rapidly while the experimental group declined mildly. The curve of E_{80} group declined rapidly at the first stage, but at the last stage the curve become less than the control, E_{10} and E_{20} group.

showed that at the begining of experiment, feeding stuff intake of each group was equal. As treatment progressing, feeding

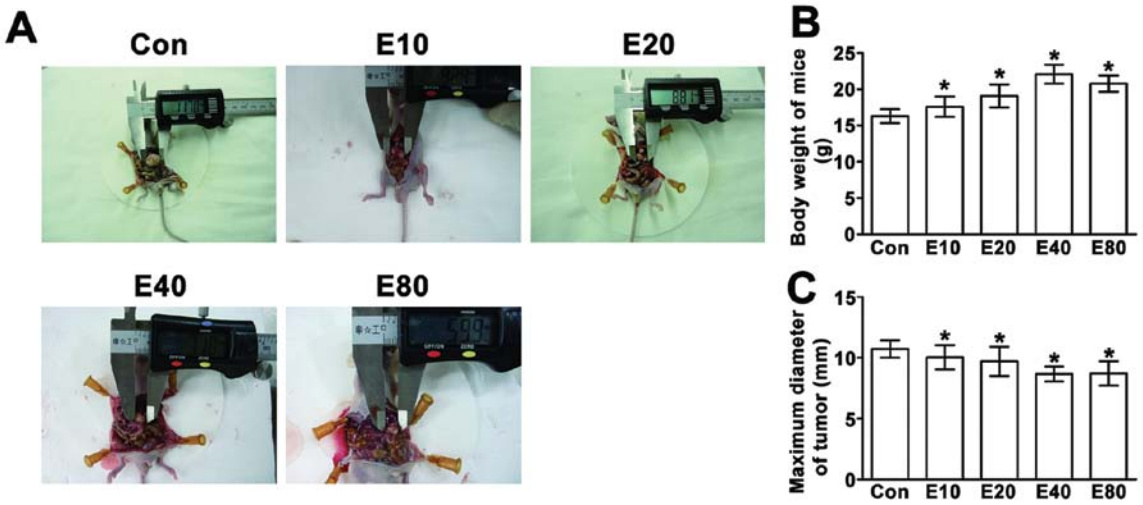

Figure 5. Effect of various concentrations of emodin on tumor growth and body weight of nude mice. (A) Images of orthotopically implanted pancreatic tumors one week after the last treatment. Emodin significantly inhibited tumor growth. (B) The average body weight of individual groups of mice one week after the last treatment. (C) The average tumor diameter of individual groups of mice one week after the last treatment. *P<0.05, compared to the control group.

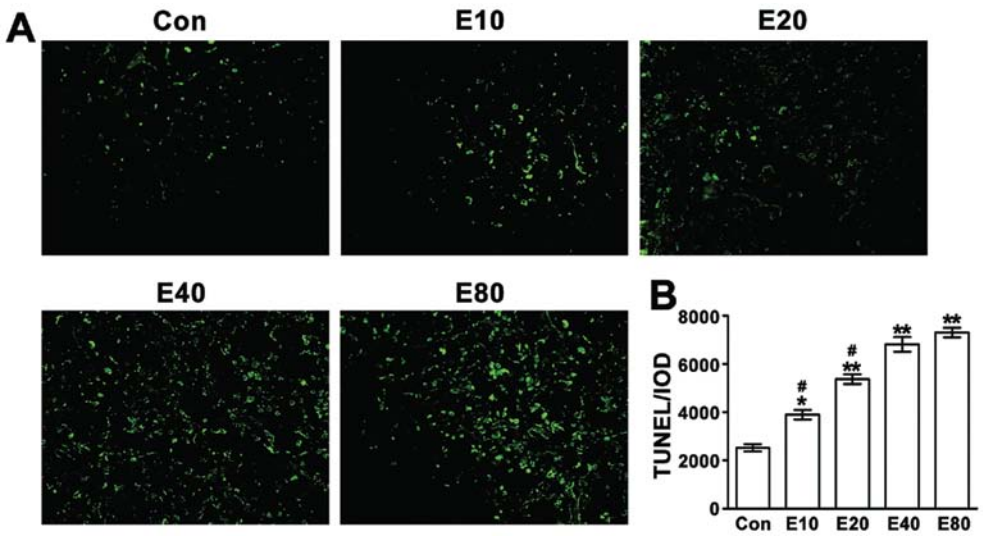

Figure 6. The effect of various concentrations of emodin on the apoptosis of implanted tumor cells. (A) Increased TUNEL-positive cells were found in the emodin treatment group (magnification, x400). TUNEL-positive cells of the tumor have an overlay of green fluorescence by the TUNEL staining. (B) TUNEL staining was further quantified and presented as average IOD level. *P<0.05, compared to the control group; **P<0.01 compared to the control groups; #P<0.05, compared to E_{40} group.

stuff intake of all groups decreased day by day. But the feeding stuff intake of E_{40} group declined less than the other groups. In the initial stage, feeding stuff intake of E_{80} group dramatically declined, but it changed gently in the last stage (Fig. 4).

Emodin inhibits the growth of tumor and increased the body weight of model mice. Compared to E_{40} and E_{80} groups, body weight in other groups was obviously reduced. The tumor diameter of E_{80} group was smaller than E_{40} group, but the body weight is smaller than E_{40} group (Fig. 5).

Emodin induces Panc-1 cell apoptosis in mouse tumors. Apoptosis of the tumor cells was detected by TUNEL assay. TUNEL-positive cells were observed with laser scanning confocal microscope (magnification, x400) (Fig. 6). With the increasing dose of emodin, the cell apoptosis increased. Apoptosis of tumor cells in E_{40} group and E_{80} group was significantly increased compared to the control group (P<0.01).

Discussion

Traditional Chinese medicine (TCM) used for tretment of cancer still have several controversies, unclear treatment mechanism and lack of theoretical basis of combination therapy (7,8). Up to now, only few kinds of TCM are accredited and applied in international society. Even so, a survey report shows that approximately 40% of American cancer patients take TCM (9).

In recent years, more and more Chinese medicine monomers are extracted. These drugs are of high purity, with single

chemical properties, such as emodin. Emodin is a kind of kinase inhibitor II (10), which could combine with DNA and prevent the proliferation and differentiation of tumor cells (11). Previous studies suggest that emodin may downregulate the nuclear factor-κB (NF-κB) that could improve the proliferation, inhibit apoptosis, enhance cancer cell drug-resistance (12) and decrease the Bax/Bcl-2 ratio (13).

Mitochondria is not only the cells' energy factory, but also a 'suicide weapon' store because multiple apoptosis pathways stay there. Changing the tumor cells' metabolism of mitochondria or stimulating the MMP decline can significantly improve the efficiency of the cancer treatment (14). Mitochondria disorders are proved to be an important part of cell apoptosis, leading to many physiological changes (15). For example, the MMP changes early when the cell is interfered with by the apoptosis factor. The inner mitochondrial membrane permeabilization causes disruption of MMP (16). In recent years, many experiments suggest that keeping the MMP steady could prevent cells from tending to apoptosis (17) and once the MMP dissipates, the cell apoptosis will be irreversible (18). Keeping MMP normal is necessary for mitochondrial function. The decline of MMP is associated with the mitochondrial membrane permeability (MPT) changing, and MPT results from the opening of a mitochondrial permeability transition pore, also known as the MPT pore or MPTP (a protein pore that is formed in the inner membrane of the mitochondria). MPTP allows water and solutes to come into the matrix, causing the expansion of the mitochondria which results in the rupture of outer mitochondrial membrane (MOM) so that the apoptosis factors such as cyt c, Smac/DIABLO, AIF, release to cytoplasm, finally leading to cell apoptosis (19,20). Our study showed that even a low concentration of emodin led to MMP decline. As the drug concentration increased, the MMP declined, with the cell apoptosis rate and cell proliferation inhibition rate rising. Our results suggest that emodin could induce MMP decline in Panc-1 cells, thereby leading to cell apoptosis and cell proliferation inhibition.

Gemcitabine was used to treat advanced pancreatic cancer as a standard drug. Most patients develop resistance to gemcitabine (21), which has strong toxic side effect and is very expensive. The success of this treatment is poor and overall survival has not improved for decades (22). In recent years, much attention was paid to Chinese traditional medicine monomers (CTMM). CTMM promotes apoptosis, inhibits proliferation, reverses drug-resistance in chemotherapy and improves the effect of chemotherapy on cancer therapy (23). Emodin is one of these drugs which has been shown to play a therapeutic role in the gastrointestinal tract, and genitourinary cancer (5,24-26). Our study showed that emodin suppressed tumor growth and induced tissue cell apoptosis. Emodin at the dose of 80 mg/kg still did not show strong drug toxicity as no unceasing decline of appetite of mice in this group was seen. At the end the body weight was more than control, E_{10} and E_{20} groups demonstrating that emodin may inhibit tumor growth and the mice can tolerate the drug toxicity.

In conclusion, our study suggests that emodin has a good effect on declining the MMP of Panc-1 cells, promoting apoptosis and inhibiting cell proliferation. This drug improved the life quality of the mice with implanted tumors.

Acknowledgments

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