Abstract. Gemcitabine is currently the best treatment available for pancreatic cancer, but causes high toxicity. Agents that can enhance the effects of gemcitabine with no or low toxicity are needed for the treatment of pancreatic cancer. Emodin, a natural anthraquinone derivative, is one such agent that has been shown to induce apoptosis in other tumor cells via down-regulation of Bcl-2/Bax and promoting the release of Cytochrome C (CytC), but with very low toxicity. The aim of this study was to evaluate whether emodin can enhance the effect of gemcitabine on pancreatic cancer in vitro and in vivo and to investigate the possible mechanisms of the enhancement. In vitro, emodin inhibited the proliferation of the SW1990 cell line and potentiated the apoptosis induced by gemcitabine, which was demonstrated by activation of caspase-3 in the combination group. In vivo, tumors from nude mice subcutaneously injected with SW1990 cells and treated with a combination of emodin (40 mg/kg) and gemcitabine (80 mg/kg) showed significant reductions in volume, Ki-67 proliferation index and expression of the Bcl-2/Bax ratio (compared with tumors from mice treated with sodium chloride, emodin alone (40 mg/kg) or gemcitabine alone (125 mg/kg), which induced increasing release of CytC from the mitochondria to the cytoplasm and triggered caspase-3 activation leading to apoptosis. Taken together, our results suggest that emodin improved the anti-tumor effect of gemcitabine, even at a lower dose of gemcitabine which could decrease the toxicity of chemotherapy, on transplanted tumors of the SW1990 cell line through the enhancement of apoptosis induced by gemcitabine, the mechanism of which may be through down-regulation of the Bcl-2/Bax ratio and promoting release of CytC from the mitochondria into the cytoplasm.

Introduction

Pancreatic cancer is a highly malignant tumor in alimentary system with very poor prognosis. One-year survival rate of patients with advanced pancreatic cancer was 8%, and 5-year survival rate was 3% (1), with the median overall survival of only 6 months (2). Complete resection of the tumor is currently the only curative option but only 10-15% of patients present with localized, potentially resectable disease at the time of diagnosis (3). Previous research shows that gemcitabine, as a DNA nucleotide analogue, has a good effect on many solid tumors including pancreatic cancer, and as an adjuvant drug after curative-intent resection, can prolong disease-free survival to at least 6 months (4). Therefore, gemcitabine was used to treat advanced pancreatic cancer as a standard drug (5,6). However, the effect of gemcitabine was limited with a low remission rate of about 10% (7,8), and median overall survival by adjuvant medication with gemcitabine after operation was only 22.1 months (4). Most patient would develop resistance to gemcitabine, which contains strong toxic side effect and is very expensive. Previous studies discovered many types of drugs that can strengthen the therapeutic effect of gemcitabine on pancreatic cancer (9-12). For the above reasons, it is valuable to find a drug which can potentiate the effect of gemcitabine but with low toxic effect and price. Emodin (1,3,8-trihydroxy-6-methylnanthraquinone) is a natural anthraquinone derivative isolated from Rheum palmatum L. Previous studies have demonstrated that emodin can inhibit cell growth and induce apoptosis in several types of tumor cells. Emodin, with low toxicity to normal cells, can induce tumor cells to produce reactive oxygen species (ROS), which results in down-regulation of Bcl-2 gene and up-regulation of Bax, leading to release of CytC from mitochondria, finally resulting in more tumor cell apoptosis (13,14).

In this study, based on in vitro cell culture and transplanted tumor in athymic nu/nu mice, we investigated our hypothesis that emodin could significantly enhance the anti-tumor effect of gemcitabine on pancreatic cancer in vivo via CytC-regulated apoptotic pathway.
Materials and methods

Cell lines and animals. The human pancreatic cancer cell line SW1990 was purchased from American Type Culture Collection. Female athymic BALB/c nu/nu mice (4–6 weeks old) were purchased from Shanghai Cancer Institute for tumor implantation. All animals were maintained in a sterile environment and cared for within animal experiment center of Wenzhou Medical College.

Drug and agents. Emodin was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO was <0.1%. Gemcitabine was purchased from Eli Lilly, dissolved in 0.9% sodium chloride. Rabbit anti-human anti-Bax, anti-Bcl-2 polyclonal antibodies, and anti-cytochrome C (CytC), anti-active caspase-3 monoclonal antibodies were bought from Abcam. In situ cell death detection kit was purchased from Roche, Germany.

Cell culture. The human pancreatic cancer cell lines SW1990 was maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin in a humidified incubator containing 5% CO₂ in air at 37°C.

Cell growth assay. To assess the viability of cells, cell numbers were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Sigma, St. Louis, MO, USA), performed according to the method of Gerlier and Thomasset (15). Briefly, cells were plated at a density of 5x10⁴ 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 final concentration of DMSO was <0.1%. A part of the tumor tissue was stained with anti-Ki-67 (rabbit monoclonal clone sp6; neoMarkers, Fremont, CA) antibody and Thomasset (15). Briefly, cells were plated at a density of 1x10⁶ cells ± SE per field (x400 magnification).

Cell apoptosis assay. After cells had been exposed to gemcitabine (20 µmol/l) for 48 h, emodin (40 µmol/l) for 48 h or their combinations (40 µmol/l emodin coincubated with 20 µmol/l gemcitabine) for 48 h at 6-well plates, SW1990 cells were washed, harvested and counted. Cells (1x10⁶) were re-suspended in 100 µl binding buffer, before 10 µl of Annexin V and 5 µl of PI were added, and incubated in the dark for 15 min at room temperature, according to the manufacturer's instruction (Biosea, China). The apoptosis rate (%) was determined with a cytometer (Epics AltraII, Beckman Coulter, USA). Cells were also viewed under an inverse fluorescent microscopy. Experiment was repeated thrice.

Ki-67 immunohistochemistry. Formalin-fixed, paraffin-embedded sections (4 µm) were stained with anti-Ki-67 (rabbit monoclonal clone SP6; NeoMarkers, Fremont, CA) antibody as described by Guha et al (16). Results were expressed as percentage of Ki-67⁺ cells ± SE per field (x400 magnification). A total of 10 fields (x400) was examined and counted from three tumors of each of the treatment groups.

TUNEL assay detect tumor apoptosis. To assess the degree of tumor apoptosis, we used the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method after two weeks of therapy. TUNEL staining was performed by standard methods with 4-µm tissue section. Operated as shown in TUNEL kit steps, tissue section was added to TUNEL reaction solution at 37°C for 1 h. Laser scanning confocal microscope under 400-fold observation camera was used, with excitation wavelength 488 nm and emission wavelength 568 nm. We observed 10 field vision of strongest fluorescence on each slice.
**Immunohistochemistry detection of apoptosis-related proteins: Bax, Bcl-2, Cytc, active caspase-3.** Sections were cut from paraffin embedded pancreatic cancer tissues. Immunostaining was performed using primary antibodies specific for Bax, Bcl-2, Cytc and active caspase-3 with appropriate dilutions, followed by staining with appropriate HRP-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counterstained with a weak solution of haematoxylin solution stain. The stained slides were dehydrated and mounted in permount and visualized on a microscope. Images were captured with an attached camera linked to a computer.

**Isolation of mitochondria and cytoplasm of tumor tissue.** Fresh tumor tissue were homogenated and then transferred to centrifuge tube, 800 x g centrifugation for 5 min at 4°C. Supernatant were collected and transferred to a new centrifuge tube. After 800 x g centrifugation for 5 min at 4°C, we discarded the precipitate. The supernatant was transferred to a new centrifuge tube. After 12,000 x g centrifugation for 10 min at 4°C, the supernatant containing cytoplasmic components and the sediment containing mitochondrial were separated and stored in -80°C until use for detecting Cytc in them by Western blot analysis.

**Western blot detection of apoptosis-related protein in tumor tissue: Bax, Bcl-2, Cytc and active caspase-3.** Proteins were routinely extracted from tumor tissues using radioimmuno-precipitation assay (RIPA) buffer. The protein concentration was detected by bicinchoninic acid (BCA) assay. The proteins were then fractionated by SDS-PAGE, electrotransferred to PVDF membranes, blocked with 5% non-fat milk, and then probed with primary antibodies and HRP-conjugated anti-rabbit secondary antibody. After washing, the bound antibody complexes were detected using ECL chemiluminescence reagent (Amersham).

**Statistical analysis.** Data are expressed as the means ± SD. Statistical analysis was performed using SPSS software. Differences in mean values between groups were determined by one-way analysis of variance followed by two-tailed Student’s t-test for unpaired samples, assuming equal variances. P<0.05 was considered statistically significant.

**Results**

**Emodin potentiates growth inhibition induced by gemcitabine in pancreatic cancer cells.** We investigated the effect of combination of emodin and gemcitabine on cell viability by MTT assay. For these studies, SW1990 cells were treated with gemcitabine (20 µmol/l) for 48 h, emodin (40 µmol/l) for 48 h or their combination [emodin (40 µmol/l) coincubated with gemcitabine (20 µmol/l) for 48 h] and analyzed for viable cells by the MTT assay as described in Materials and methods. Data are presented as mean ± SE of nine replicates from three independent experiments. *P<0.01 compared with sodium chloride treatment; **P<0.01 compared with sodium chloride, emodin or gemcitabine treatment.

**Emodin augments the anti-tumor effect of gemcitabine in xenograft model of pancreatic cancer.** The schematic overview of the study protocol is presented in Fig. 3A. SW1990 xenografts were established in athymic mice within 3 weeks and the nude mice were randomly divided into four experimental groups (12 mice in each group), which were respectively subjected to treatment with control (0.9% sodium chloride), emodin (40 mg/kg), gemcitabine (125 mg/ kg) or emodin (40 mg/kg) plus gemcitabine (80 mg/kg) every three days. At the beginning of the therapy, the tumor volume of all mice was (0.38±0.05) cm³, with no significant difference among groups (P>0.05). Emodin, gemcitabine or emodin plus gemcitabine treatments dramatically suppressed tumor growth and combination drug therapy was more effective in tumor growth suppression compared to single agent treatment.
inhibition than mono-therapy with either agent (Fig. 3B). One week after the final injection, mice were sacrificed and solid tumors were presented (Fig. 3C). Consistent with in vitro results, both tumor weight and volume were suppressed by mono-therapy with either agent or combination therapy with emodin plus gemcitabine compared with control, while combination treatment exhibited more efficient than single treatment (P < 0.05) (Table I). These results demonstrated that emodin and gemcitabine combination treatment showed efficient tumor growth suppression in pancreatic tumor inoculated mice.

**TUNEL staining shows emodin potentiates tumor cell apoptosis by gemcitabine.** We next examined the expression of the cell proliferation marker Ki-67. The results in Fig. 4A and B show that emodin in combination with gemcitabine
significantly down-regulated the expression of Ki-67 in tumor tissues compared with other groups (P<0.05). As apoptosis is one major pathway which leads tumor cells to death, we investigated apoptotic cell death induced by drug treatment. In vivo, we found inhibition of cell growth was also correlated with apoptotic cell death. As shown in Fig. 4C, increased TUNEL-positive cells were observed in drug therapy groups, and combination treatment remarkably promoted the apoptotic cell death. Quantified data revealed that combination therapy with emodin plus gemcitabine dramatically elevated integrated optical density (IOD) as compared with other groups (P<0.01) (Fig. 4D). Above results were consistent with our in vitro results.

Immunohistochemistry shows combination treatment reduces Bcl-2/Bax ratio, promotes caspase-3 activation and CytC release. Decreased Bcl-2/Bax ratio, activation of caspase-3 and CytC release are recognized as the key events that mediate the apoptotic pathway. To clarify the mechanism of emodin plus gemcitabine-promoted apoptotic cell death in tumor tissues, the expression of anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax, active caspase-3 and CytC were assessed by immunohistochemistry. As revealed in Fig. 5, reduced Bcl-2/Bax ratio, but enhanced caspase-3 activation and CytC release from mitochondrial were observed after drug injection (P<0.01 compared with control). Importantly, combination therapy further promoted the reduction of Bcl-2/Bax ratio (P<0.01 compared with monotherapy with either agent) (Fig. 5B). The ratio of Bcl-2/Bax is essential for regulating CytC release from the mitochondria as well as caspase-3 cleavage. Immunohistochemical analysis confirmed the elevated cytosolic CytC level and activated caspase-3

Table I. Effect of combination treatment on tumor growth inhibition.

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug dose (mg/kg)</th>
<th>Tumor weight (g)</th>
<th>Tumor volume (cm³)</th>
<th>Inhibition rate (%)</th>
</tr>
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<tbody>
<tr>
<td>Sodium chloride</td>
<td>0</td>
<td>2.38±0.15</td>
<td>2.45±0.16</td>
<td>0</td>
</tr>
<tr>
<td>Emodin (E)</td>
<td>40</td>
<td>1.68±0.13</td>
<td>1.66±0.11</td>
<td>37.5</td>
</tr>
<tr>
<td>Gemcitabine (G)</td>
<td>125</td>
<td>1.32±0.05</td>
<td>1.20±0.12</td>
<td>59.1</td>
</tr>
<tr>
<td>(E)+(G)</td>
<td>40 (E)+80 (G)</td>
<td>0.56±0.07</td>
<td>0.57±0.08</td>
<td>90.8</td>
</tr>
</tbody>
</table>

One week after the final injection (Day 37), mice were sacrificed, and tumor weight was measured. Tumor volume and inhibition rate were calculated as described in Materials and methods. *P<0.05 compared with sodium chloride treatment; **P<0.05 compared with sodium chloride, emodin or gemcitabine treatment.
protein expression in the combination treatment group (P<0.01 compared with mono-therapy or with either agent) (Fig. 5C).

**Further detection of Bcl-2/Bax ratio, caspase-3 activation and CytC release.** Similar results to the above in immunohistochemistry were obtained from Western blot analysis (Fig. 6). Our results of Western blotting show obviously reduced Bcl-2/Bax ratio and strong caspase-3 activation by combination of emodin and gemcitabine (Fig. 6A-C), thus, we assessed CytC release into the cytosol mitochondrial extract and mitochondria-free cytosolic extract by immunoblotting (Fig. 6A). A significant decrease in the mitochondrial CytC level in the combination treatment was observed compared with other groups, while appearance of CytC in the cytosol was significantly up-regulated compared with other groups (Fig. 6D). These data demonstrated combination therapy with both emodin and gemcitabine promoted tumor cell apoptosis via down-regulation of Bcl-2/Bax ratio, activation of caspase-3 and CytC release from mitochondria to the cytosol.

**Discussion**

In recent years, research of using natural substances to enhance the chemotherapeutic effect of gemcitabine was widespread. A number of studies have shown that natural compounds can enhance the therapeutic effect of gemcitabine on pancreatic cancer (17-19). Emodin can inhibit the growth of various cancer cells, including pancreatic cancer (20-22), and has been shown to increase sensitivity of paclitaxel, cisplatin, and etoposide, doxorubicin, celecoxib and arsenic trioxide and other drugs inducing apoptosis (23-25), including significantly enhanced anti-tumor and pro-apoptotic effect of gemcitabine on pancreatic cancer Panc-1 and Bxpc-3 cells (26). But the study in vivo on the mechanism of the enhanced
effect by combination of emodin and gemcitabine was absent. For the first time, we found, in vivo, emodin sensitized pancreatic cancer cell line SW1990 to gemcitabine through down-regulation of Bcl-2/Bax ratio and increasing release of CytC from mitochondria to cytosol. And interestingly, we first found that emodin enhanced the effect of gemcitabine on pancreatic cancer in vivo, even if the dose of gemcitabine was reduced.

Consistent with previous study by Guo et al (26), we also found in vitro emodin can sensitize pancreatic cancer to apoptosis induced by gemcitabine. Furthermore, we firstly found similar results in in vivo experiments, as evidenced by TUNEL assay and analysis of caspase-3 activation by immunochemistry and immunoblotting. We subsequently investigated the mechanism of how emodin potentiated the pro-apoptotic effect of gemcitabine in vivo. An upstream event in activation of the caspase-3 is the release of CytC from mitochondria which can trigger caspase-3 leading to apoptosis. Many cancer-preventive and chemotherapeutic agents have been shown to activate the apoptotic pathway that involves the release of CytC from mitochondria, which then oligomerizes with procaspase-9 leading to formation of active caspase-9 that activates downstream executioner caspases such as caspase-3 and caspase-7 (27). It has been reported that emodin can down-regulate Bcl-2 expression, up-regulate Bax level, trigger CytC release, and subsequently activate
caspases-2, 3, 9, and induce apoptosis in other kinds of cancer cells (15,16). Our results are consistent with these reports, showing increased levels of active caspase-3 in combination group of emodin and gemcitabine, what is more, concomitant with a decrease in mitochondrial CytC which was further confirmed by the increase in cytosolic CytC in combination group compared to control or single agent groups. These results suggested that emodin enhances the pro-apoptotic effect of gemcitabine on subcutaneously transplanted pancreatic cancer by regulating CytC release.

The balance of pro-apoptotic and anti-apoptotic proteins, such as the Bcl-2/Bax ratio, is essential in cell apoptosis, and alteration of this balance allows sensitization to apoptotic effect of chemotherapy. Bax up-regulation in breast (28) and pancreatic cancer (29) is associated with longer survival, which seems to be related to Bax homodimer formation or an imbalance in the Bcl-2/Bax ratio towards Bax, which promotes cell death (30-32). Furthermore, Bcl-2 and Bax respectively suppresses and promotes CytC to release from mitochondria, so we further investigated the variation of Bcl-2/Bax ratio.

In this study, we observed a remarkably decreased Bcl-2/Bax ratio in tumor tissues after combination treatment as revealed by immunohistochemical and Western blot analysis, demonstrating emodin enhances the pro-apoptotic activity of gemcitabine in pancreatic cancer cells via down-regulating the Bcl-2/Bax ratio. Our study revealed that emodin might be able to down-regulate Bcl-2 expression, up-regulate Bax level, triggers the CytC release, and subsequently activates caspase-3 and induces cell apoptosis.

Several randomized studies have shown significant increase in patient response rate by the use of combinations of different class of chemotherapeutic agents, but the major problem is due to treatment associated high toxicity with no added benefit in overall survival (33-35). However, these limitations could be overcome by the use of rational chemotherapeutic combinations, in which toxic agents are used in lower doses, and the efficacy of treatment is complemented by using a non-toxic agent. Based on this...
rationale, we used emodin in combination with gemcitabine to test its efficacy against pancreatic cancer transplanted tumors. Our results showed that lower dose of gemcitabine combined with emodin exhibited enhanced suppression of tumor growth compared to high dose of gemcitabine therapy. This result suggested emodin combined with gemcitabine might be a new effective and low toxic therapeutic strategy for pancreatic cancer.

In summary, this study showed that emodin combined with lower dose of gemcitabine can significantly enhance the anti-tumor effect of gemcitabine on pancreatic cancer SW1990 cell transplanted tumors through inducing apoptosis of tumor cells, the mechanism of which may be that emodin decreases Bcl-2/Bax ratio, and then causes the release of Cytc, from mitochondria into cytoplasm, thus leading to more significant apoptosis of tumor cells via activation of caspase-3. However, further mechanistic studies could be useful to fully support our strategy for the treatment of patients with pancreatic tumors.

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References