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Full Length Research Paper

Parthenolide augments the anticancer effects of 5-fluorouracil on cell apoptosis and decreased NF-κB activity in a gastric cancer cell line

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5-Fluorouracil (5-FU) is not well tolerated by some patients, because of its severe side effects. The aim of the present study was to investigate the effect of parthenolide (PTL) in augmenting the anti-tumor effect of 5-FU in gastric cancer cells KATO III and to determine if parthenolide can be used to decrease the dose of 5-FU. The mechanism underlying the anti-tumor effects of 5-FU was also investigated. Using flow cytometry, we analyzed apoptosis of KATO III cells. Growth proliferation of the cell line was assayed by MTT. PGE_2 protein level was detected by ELISA and western blot analysis was used to detect the expression of the NF-κB/p65, COX-2, caspase-3 and caspase-9 proteins. We found that either 5-FU or PTL alone induced apoptosis of KATO III cells in a concentration-dependent manner; however, combined treatment with both 5-FU and PTL significantly improved apoptosis. MTT data demonstrated that KATO III cells were more sensitive to 5-FU treatment in the presence of PTL. The combination of 5-FU with PTL also resulted in a significant reduction of PGE_2 secretion, compared with the control. Expression of NF-κB/p65 and COX-2 was inhibited, whereas, caspase-3 and caspase-9 protein were upregulated. In conclusion, it is rational to include the NF-κB inhibitor PTL in regular 5-FU-based chemotherapy, which may not only allow a reduction in the dose of 5-FU to prevent adverse effect, but also enhance the chemotherapeutic effect of 5-FU in gastric cancer.

Key words: Parthenolide, 5-fluorouracil, gastric cancer, apoptosis, nuclear factor-kappa B.

INTRODUCTION

Digestive tract cancer, including gastrointestinal carcinoma, is characterized by its low diagnosis rate at early stages. Often, the diagnosis is made too late for radical surgery to be feasible and palliative surgery and chemotherapy are the only options for management. 5-fluorouracil (5-FU), one of the most widely used chemotherapeutic drugs for gastrointestinal carcinoma, is not tolerated by some patients, because of its severe side effects (Ciccolini et al., 2006; Lazar et al., 2004; van Kuilenburg et al., 2003). In addition, many patients show resistance to 5-FU after long-term exposure to this drug, and tumors recur. So, choosing the right dosing scheme and chemotherapy strategy for each individual patient, while minimizing side effects, remains a challenge for individual-based and personalized chemotherapy management (Ciccolini et al., 2004). Meanwhile, new drugs that can efficiently augment the anti-tumor effect of 5-FU, allowing a reduction in the dose of 5-FU, are urgently needed.

Parthenolide (PTL), which is the major sesquiterpene lactone isolated from extracts of Mexican Indian medicinal plants such as feverfew (*Tanacetum parthenium*), has been used for several centuries to treat migraines, rheumatoid arthritis and inflammation (Pozarowski et al., 2003). Recently, it has been reported

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that PTL inhibits proliferation and induces apoptosis of various human cancer cell lines, such colorectal cancer, hepatoma, cholangiocarcinoma and primary acute/chronic myeloid leukemia cells *in vitro* (Wang et al., 2009; Park et al., 2005; Kim et al., 2005; Guzman et al., 2005; Steele et al., 2006). In addition, PTL enhances the sensitivity of resistant cancer cells to other anti-tumor agents (Zhang et al., 2004; Nakshatri et al., 2004) and acts as a chemopreventive agent in an animal model of UVB-induced skin cancer (Won et al., 2004). Further research has shown that PTL-induced apoptosis is associated with inhibition of the anti-apoptotic transcription factor nuclear factor-kappa B (NF-κB) activity (Pozarowski et al., 2003; Kishida et al., 2007). It has been reported that high expression of NF-κB can exert not only anti-apoptotic effects, but is also involved in the mechanism of resistance to many drugs, including 5-FU. Thus we hypothesized that PTL might have the ability to augment the anti-tumor effect of 5-FU through inhibition of NF-κB activity in gastric cancer cells. In this study, we analyzed the effect of 5-FU alone and in combination with PTL on the proliferation and apoptosis of the human gastric cancer cell line KATO III. We also investigated several downstream molecular mechanisms.

MATERIALS AND METHODS

Drugs and materials

Parthenolide (Sigma, St Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) and diluted to a final concentration of 10, 50, 100, 200, 500, or 1000 μM. 5-FU (Shanghai, China) was dissolved in saline solution and diluted to 0.5, 1, 2, 5, 10 or 20 μg/ml. Annexin V-FITC Kit was purchased from Bender MedSystems GmbH (Vienna, Austria). Antibodies used in western blot analysis were: rabbit anti-active caspase-3 and caspase-9 (Chemicon, CA, USA), which recognize only the cleaved large subunit (17 kDa of caspase-3 and 37 kDa of caspase-9) and rabbit anti-cyclooxygenase-2 (COX-2), NF-κB/p65 and β-actin polyclonal primary antibodies (Santa Cruz, California, USA). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody.

Cell culture and treatments

Human gastric cancer KATO III cells were obtained from American Type Culture Collection (Rockville, MD). KATO III cells were cultured in RPMI 1640 (Gibco BRL, N.Y., USA) containing 10% fetal bovine serum (Hyclone, Utah, USA) in an air incubator containing 5% CO_2 at 37°C. When the adherent cells became confluent, cells were treated with PTL alone (10, 50, 100, 200, 500 or 1000 μM), 5-FU alone (0.5, 1.0, 2.0, 5.0, 10 or 20 μg/ml) or PTL (10, 100, 500 μM) in combination with a much lower concentration of 5-FU (2.0 μg/ml), for 48 h.

Flow cytometry analysis of apoptosis

The effect of the drugs on apoptosis of KATO III cells was investigated by flow cytometry assay. An Annexin V-FITC/PI apoptosis detection kit was used according to the manufacturer's instructions to detect typical apoptosis and necrosis. Briefly, suspensions were washed twice and adjusted to a concentration of 1×10^6 cells/ml with ice-cold PBS. Next, they were double-stained with Annexin V-FITC and propidium iodine for at least 20 min at room temperature in the dark, after which PBS binding buffer was added without washing. Flow cytometry was performed with a 488-nm laser coupled to a cell sorter (FacsCalibur, BD Biosciences, San Jose, CA, USA). Cells stained with both propidium iodide and Annexin V-FITC was considered necrotic and cells stained only with Annexin V-FITC were considered apoptotic.

Growth proliferation assay

The effect of the drugs on growth inhibition of KATO III cells was determined by detecting 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Sigma) dye absorbance of living cells. Briefly, KATO III cells (1×10^5 cells per well) were seeded in 96-well plates. After exposure to the different treatments for 48 h, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well and the plates were incubated for an additional 4 h at 37°C. The MTT solution in the medium was then aspirated off. To achieve solubilization of the formazan crystals formed in viable cells, 150 μl of DMSO was added to each well before absorbance at 570 nm was detected. Each group included six parallel wells. We repeated this assay three times.

Assay for PGE_2 production

The levels of PGE_2 in single- or combination-treated culture supernatants were determined using a competitive enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, MN, USA) according to the manufacturer's instructions.

Western blot assay

After treatments, cells were collected and washed three times with ice-cold PBS. Cell lysates were prepared for western blot analysis of NF-κB/p65, COX-2, caspase-3, caspase-9 and β-actin, using whole cellular protein extraction kits (Active Motif, California, USA). The protein concentration in each cell lysate was determined using a DC protein assay kit (Bio-Rad, Richmond, CA, USA). Protein was mixed with 2×SDS sample buffer. Proteins (40 μg) were separated in a 10% polyacrylamide gel and blotted on a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes were blocked with 5% BSA (Sigma) in TBS (25 mM Tris-HCl, 150 mM sodium chloride and pH 7.2) for 2 h at room temperature.

Blots were incubated with anti-NF-κB/p65, anti-COX-2, anti-caspase-3 and caspase-9 or anti-β-actin specific rabbit polyclonal IgG primary antibody at a 1:500 dilution overnight at 4°C. Blots were washed three times in washing buffer (PBS with 0.1% Tween-20) and then incubated in HRP-conjugated goat anti-rabbit antibody (1:2000 dilution) for 2 h at room temperature. All blots were developed using the ECL chemiluminescence detection system (Supersignal Dura kit, Pierce) following the manufacturer's instructions. Blots were scanned and analyzed for measurement of the band intensities. In all experiments, Ponceau staining was carried out to control equal loading.

Statistical analysis

The results are expressed as the mean value and standard error of the mean. Statistical significance was analyzed by one-way analysis of variance. $P < 0.05$ was considered statistically significant.

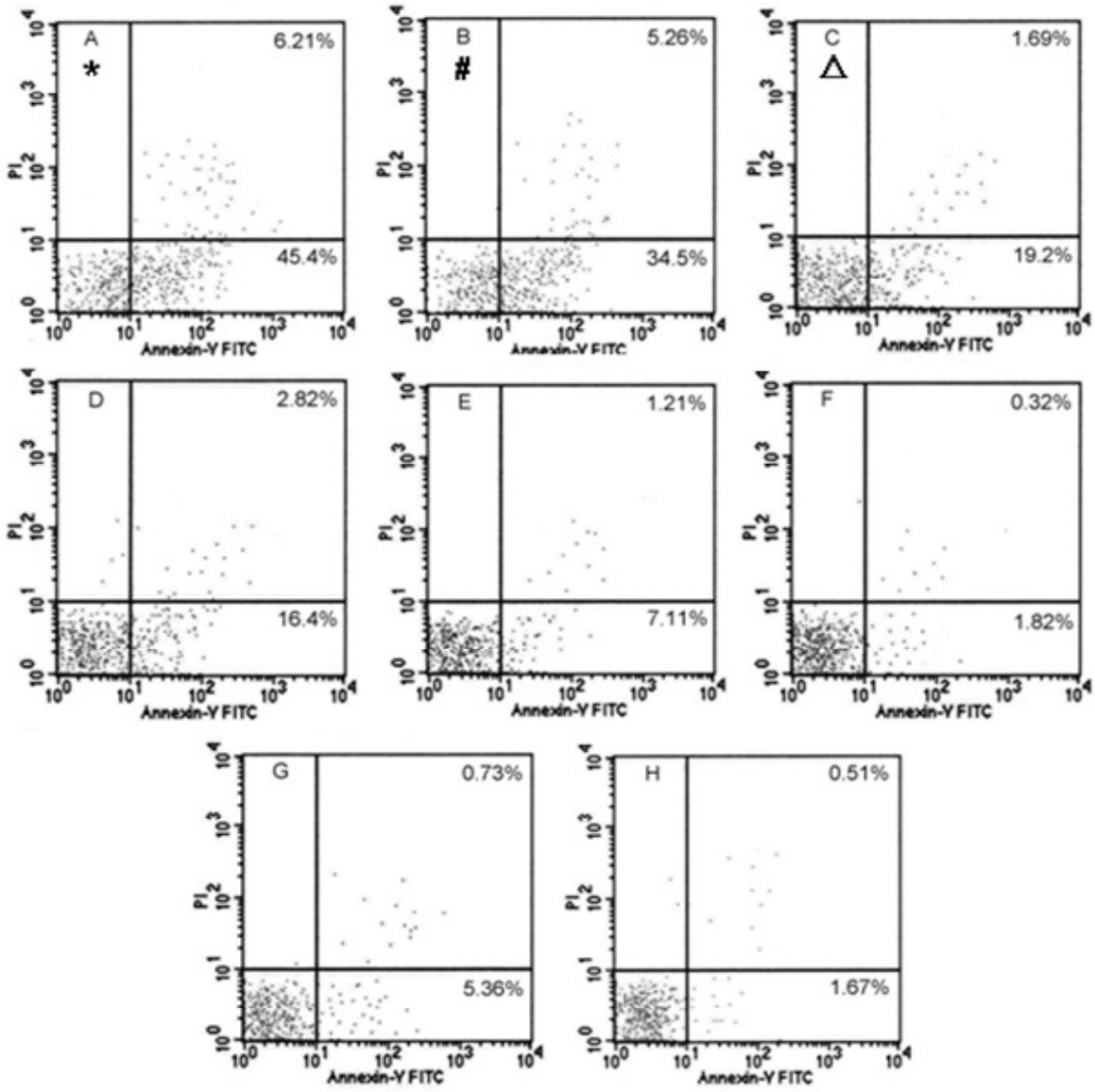

Figure 1. Effect of PTL and 5-FU on induction of apoptosis of KATO III cells. Flow cytometry results showed that there is significant increase in the apoptosis rate of KATO III cells in when 5-FU was combined with PTL, compared with PTL alone (P <0.05). This effect was manifested in a concentration-dependent manner. Representative data are shown. A: PTL 500 µM + 5-FU 2 µg/ml, B: PTL 100 µM + 5-FU 2 µg/ml, C: PTL 10 µM + 5-FU 2 µg/ml, D: PTL 500 µM, E: PTL 100 µM, F: PTL 10 µM, G: 5-FU 2 µg/ml, H: normal. * P < 0.01 *vs* group D, G and H, # P < 0.01 *vs* group E, G, and H, △ P < 0.01 *vs* group F, G and H.

RESULTS

Effect of PTL and 5-FU on inducing apoptosis of KATO III cells *in vitro*

To investigate the apoptosis effect, the Annexin V-FITC and PI double-staining method was used to detect the percentage of apoptotic cells. Either 5-FU or PTL alone induced apoptosis of KATO III cells in a concentration-dependent manner. Apoptosis induced by different concentrations of 5-FU (0, 0.5, 1.0, 2.0, 5.0, 10, 20 µg/ml) was 1.44, 1.79, 2.61, 4.72, 8.26, 19.4 and 26.6%, respectively, while that induced by PTL (0, 10, 50, 100, 200, 500, 1000 µM) was 1.83, 1.96, 4.86, 7.26, 12.4, 18.6, and 22.9%, respectively.

To determine the combined effect of 5-FU with PTL, a lower concentration of 5-FU (2 µg/ml) was chosen. At this concentration, 5-FU alone did not induce apoptosis. Surprisingly, combination of 5-FU with PTL significantly improved apoptosis. Figure 1 shows that the percentage

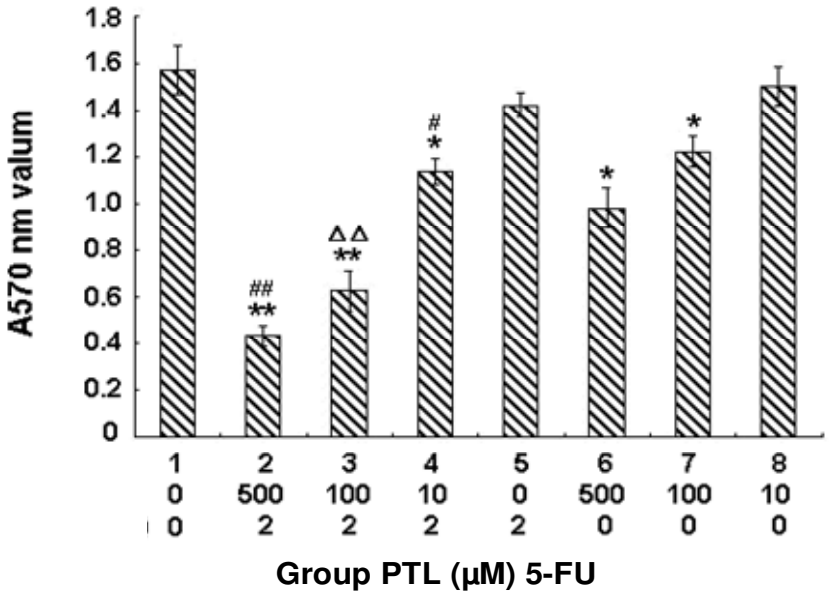

Figure 2. Effect of PTL and 5-FU on proliferation inhibition of KATO III cells. MTT assay results showed that PTL or 5-FU alone exhibited a concentration-dependent inhibitory effect, while co-application at all concentrations also significantly enhanced the anti-proliferation capability. ** $P < 0.01$ *vs* group 1, * $P < 0.05$ *vs* group 1, ## $P < 0.01$ *vs* group 6, △△$P < 0.01$ *vs* group 7, # $P < 0.05$ *vs* group 8.

of apoptotic cells was elevated significantly (representative results were 19.2, 34.5.8, and 45.4%, respectively) when 5-FU was combined with PTL (10, 100, 500 μM). This indicates a synergistic action between PTL and 5-FU. Thus, PTL might act as a regulator to down regulate the apoptosis threshold of cancer cells, resulting in increased sensitivity of KATO III cells to 5-FU.

Effect of PTL and 5-FU on proliferation inhibition of KATO III cells

MTT assay was carried out to detect KATO III cell viability after different treatments. The results show that PTL or 5-FU alone had a concentration-dependent inhibitory effect, while the anti-proliferation capability of 5-FU was significantly enhanced when combined with PTL (Figure 2). The data demonstrate that KATO III cells are more sensitive to the combined treatment. In the presence of PTL, the anti-proliferation effect of a lower concentration of 5-FU was improved.

PGE_2 protein production in culture supernatants

When KATO III cells were subjected to single or combined treatment for 48 h, the levels of PGE_2 in culture supernatants were determined by competitive ELISA. PGE_2 secretion was significantly reduced by PTL at concentrations above 10 μM. All concentrations of combined 5-FU and PTL also resulted in a significant reduction of PGE_2 secretion, compared with the control (P <0.01). Moreover, a substantial decline was observed in the 100 and 500 μM groups versus the single treatment group (P <0.05), but a low concentration of 5-FU (2 μg/ml) did not show any statistically significance effect (Figure 3).

Expression of the NF-κB/p65, COX-2, caspase-3 and caspase-9 protein

After confirming that combination of 5-FU with PTL could induce apoptosis and profoundly inhibit proliferation of KATO III cells, we attempted to investigate the potential molecular mechanism involved. Expression of NF-κB/p65 and COX-2 was examined by western blot analysis. PTL treatment alone and in combination with 5-FU for 48 h significantly inhibited the expression of NF-κB/p65 and COX-2. However, 5-FU treatment alone seemed to have no effect on NF-κB /p65 and COX-2 expression, compared with the control (Figure 4).

We also measured the expression of the cleaved forms of caspase-3 and caspase-9, which are strongly associated with cell apoptosis. As expected, the cleaved form of caspase-3 (17 kDa) and caspase-9 (37 kDa) protein were upregulated by PTL in a concentration-dependent manner. Moreover, combination of 5-FU with

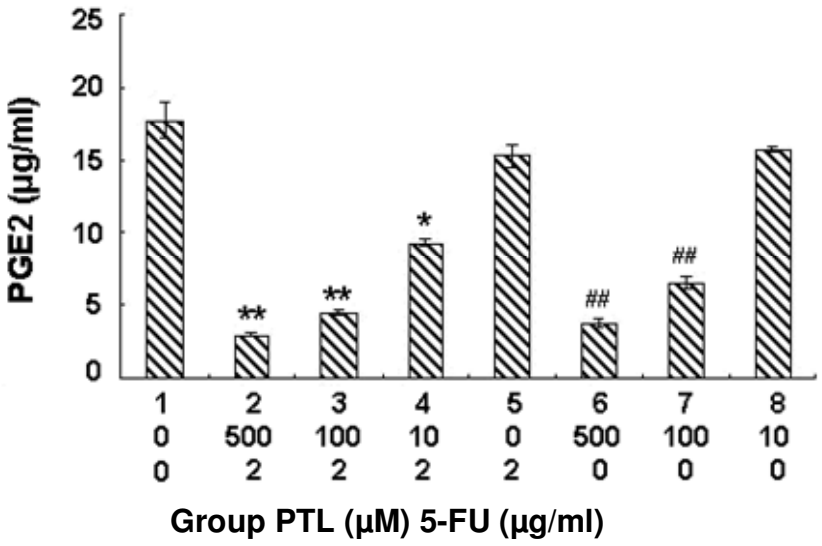

Figure 3. PGE_2 protein level in culture supernatants. PGE_2 protein level in culture supernatants was detected by ELISA. PGE_2 secretion was significantly reduced by PTL when the concentration was above 10 μM. All concentrations of combined 5-FU with PTL also resulted in a significant reduction of PGE_2 secretion, compared with the control. Moreover, a substantial decline was observed in the 100 μM and 500 μM groups, versus the single treatment group. ** $P < 0.01$ *vs* group 1 and 5, * $P < 0.05$ *vs* group 1 and 5, ## $P < 0.01$ *vs* group1.

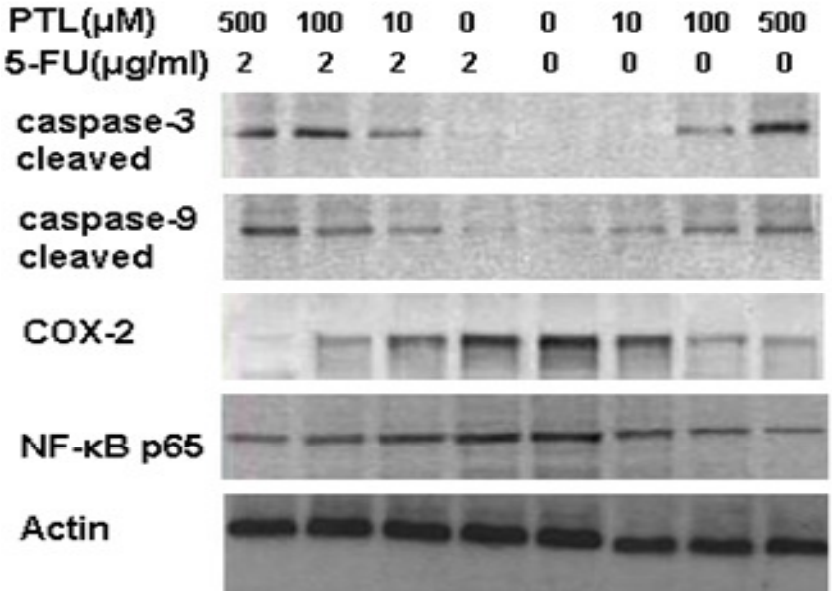

Figure 4. Expression of the NF-κB/p65, COX-2, caspase-3 and caspase-9 protein. Expression of the NF-κB/p65, COX-2, caspase-3 and caspase-9 protein was detected in gastric cancer specimens by western blot assay.

different concentrations of PTL was much more powerful in activating caspase-3 and caspase-9 than PTL alone, especially at PTL concentrations of 100 and 500 μM (Figure 4).

DISCUSSION

Gastrointestinal carcinoma is a common malignancy worldwide and very pronounced in China. Although, 5-FU

has been widely used as the first-line chemotherapy drug for the treatment of gastrointestinal carcinoma, the severe toxic reactions and high resistance to it after long-term treatment limit its clinical application. Nowadays, cancer treatment tends to be individualized. Thus, it is urgent to finds ways to reduce the adverse effects of 5-FU and prevent drug resistance, while enhancing the efficacy on cancer therapy. In our study, we found that combination of 5-FU with PTL had a much more powerful effect on induction of apoptosis, inhibition of cell proliferation and PGE_2 expression in human gastric cancer KATO III cells than either 5-FU or PTL alone. In addition, we showed that activation of NF-κB and COX-2 was down regulated after treatment with PTL alone or PTL combined with 5-FU. Caspase-3 and -9 were also significantly activated after treatment with PTL and 5-FU.

Although, surgery remains the primary treatment in the management of gastric cancer, 5-FU-based chemotherapy in conjunction with surgery represents the standard care in China. The molecular mechanism after absorption by cancer cells has been reported to involve conversion of 5-FU to deoxynucleotide, which influences DNA synthesis, or into 5-FU nucleoside, which is incorporated into RNA in the form of a pseudo-metabolic product affecting protein synthesis, which then induces tumor cell apoptosis. Our study demonstrated that 5-FU induced apoptosis of gastric cancer KATO III cells in a concentration-dependent manner. However, the chemo-therapeutic effectiveness is usually disappointing owing to multiple drug resistance induced by long-term application of 5-FU. There is evidence showing that 5-FU might activate the anti-apoptotic factor NF-κB, preventing 5-FU from killing cancer cells, which might contribute to the anti-apoptosis and drug resistance mechanisms.

NF-κB, is a ubiquitous transcriptional factor and can be detected in the cytoplasm of many cell types as an inactive complex through the direct binding of the natural inhibitor of κB (IκB). NF-κB is a major player in the control of apoptosis, cell proliferation and differentiation, and is activated in response to several pro-apoptotic stimuli, such as TNF-α, ionizing radiation, oxidative stress and cytotoxic drugs including 5-FU. All of these stimuli trigger phosphorylation and degradation of IκB and release of NF-κB, which then translocates into the nucleus and regulates the expression of anti-apoptotic genes. Thus inhibition of NF-κB in cancer cells has become one of the major targets of anticancer therapy. PTL is one of the traditional medicines extracted from the herb Feverfew in Europe and America. Studies have shown that PTL targets NF-κB by inhibiting the upstream regulator IκB kinase (IKK) (Jin et al., 2009), which phosphorylates IκB and targets it for proteasomal degradation. As a result, DNA-binding of NF-κB is blocked and cancer cells undergo apoptosis or become sensitized to anti-cancer drug-induced cell death. Therefore, it is reasonable to combine 5-FU with PTL in cancer therapy, as PTL is able to inhibit the anti-apoptotic effect of NF-κB activated by 5-FU and enhance the apoptotic effect of 5-FU. Accordingly, our study demonstrated for the first time that the synergistic effect of 5-FU and PTL on induction of cell apoptosis and inhibition of proliferation was much stronger than that of either agent alone.

In this study, we also found that some NF-κB-related genes including COX-2 and PGE_2 in KATO III cells were significantly down regulated after inhibition of NF-κB activity by PTL. COX-2, which is expressed during the early stages of adenocarcinomas of stomach, colon, breast, lung and prostate, is involved in the conversion of arachidonic acid into prostaglandins such as PGE_2 and thromboxanes, as well as the synthesis of malonaldehyde and the production of hydrogen peroxide. These products in turn promote carcinogenesis by mechanisms including transformation of procarcinogens to carcinogens, pro-inflammatory and immunomodulatory effects, resistance to apoptosis, angiogenesis, and invasion progression.

Conclusions

Taken together, our results show for the first time that PTL augments the anti-tumor effect of 5-FU through inhibition of NF-κB activity, activation of caspase-3 and caspase-9, and induction of apoptosis. Our study proved that it is rational to adopt NF-κB inhibitor PTL into regular 5-FU-based chemotherapy strategy, which may not only reduce the dose of 5-FU to prevent adverse effects, but also enhance the chemotherapeutic effect on gastric cancer.

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