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Abstract. The aim of the present study was to investigate the mechanism underlying the antitumor effects of ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F) in colorectal cancer (CRC). 5F was isolated and used to treat C26 murine colon carcinoma cells, a xenograft tumor mouse model (induced by C26 cells) and a CRC mouse model (induced by 1,2-dimethylhydrazine (DMH)/dextran sodium sulfate (DSS)). C26 cell growth was inhibited by 5F in a dose- and time-dependent manner in vitro. In addition, 5F induced cell apoptosis and cell cycle arrest in the G₁ phase, increased the activity of caspase-3 and caspase-9, but did not affect the activity of caspase-8, suggesting that 5F induced apoptosis via activation of the mitochondrial signaling pathway rather than the death-receptor signaling pathway. Furthermore, treatment of C26 cells with 5F resulted in upregulation of cyclin-dependent kinase inhibitor 1A (p21, Cip1), Bcl-2-associated X protein, nuclear factor κ-light polypeptide gene enhancer in B-cells inhibitor, α and downregulation of B-cell lymphoma 2, nuclear factor κ-light-chain enhancer of activated B cells and survivin. In vivo animal models demonstrated that 5F treatment protected mice from carcigenesis induced by DMH/DSS and markedly decreased the xenograft tumor weight with minimal side effects. Therefore, 5F may have potential as an anti-CRC therapeutic agent for use in the clinical setting.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed type of cancer worldwide (1), and is ranked the sixth and fifth in prevalence and mortality, respectively in China (2). In 2010, it was estimated that 274,841 new cases were diagnosed in China and 132,110 cases resulted in CRC-associated mortality (2). Despite significant advances in early diagnosis and therapy, the prognosis of CRC remains poor. Thus, development of effective and safe therapeutic strategies for CRC is required.

Ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F) is isolated from a traditional Chinese herb, Pteris semipinnata L. (PsL). It has been demonstrated to kill various human cancer cells, such as nasopharyngeal carcinoma, lung cancer, laryngeal cancer, thyroid carcinoma, gastric cancer and colorectal cancer cells, in vitro by inducing apoptosis (3-8). Previous studies in mice also demonstrated that 5F is effective against liver and lung cancer with minimal side effects (9,10). Few previous studies have investigated treatment of CRC via 5F-induced apoptosis. Therefore, the present study aimed to examine the antitumor effect of 5F in vitro and in vivo using CRC cells and two CRC mouse models, respectively.

Materials and methods

Cell culture. The C26 murine colon carcinoma cell line was obtained from Peking Union Medical College (Beijing, China). C26 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Beyotime Institute of Biotechnology, Haimen, China). Cells were cultured at 37˚C in 5% CO₂.

Cell proliferation assays. The 5F was isolated from PsL (Qingping Medicine Market, Guangzhou, China) as previously described (11), dissolved in propylene glycol (Sigma-Aldrich) and diluted immediately prior to use. C26 cells (5x10⁵ cells/well) were seeded in a 96-well plate in 200 µl RPMI-1640 medium. Following culture for 24 h, the
cells were exposed to 0, 5, 10, 20, 40 or 80 µg/ml 5F. Cell viability was examined at 24, 48 or 72 h using the MTT assay (Sigma-Aldrich) and expressed as a percentage of the control cells. The morphology of the cells was observed at 24 h under an inverted phase contrast microscope (CKX31-A12PHP; Olympus, Tokyo, Japan).

Cell cycle distribution. C26 cells were seeded at a density of 1x10^5 cells/well in a 6-well plate. The cells were treated with 0, 5, 10, 20 or 40 µg/ml 5F for 24 h, and fixed with 70% ethanol (Beijing Chemical Reagent Factory, Beijing, China) at -20°C overnight prior to staining with 100 µl/ml propidium iodide (PI) solution (Molecular Probes®; Thermo Fisher Scientific, Waltham, MA, USA). Cell cycle distribution analysis was performed using a Coulter EPICSXL 31240 flow cytometer, (Beckman Coulter, Inc., Brea, CA, USA).

Annexin V/PI staining. Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit (Beyotime Institute of Biotechnology). The cells were treated with 0, 5, 10, 20 and 40 µg/ml 5F for 24 h, prior to harvesting. The cells were washed once with phosphate-buffered saline and resuspended in 500 µl binding buffer (both from Beyotime Institute of Biotechnology). Annexin V-FITC solution (5 µl) and dissolved PI solution (5 µl) were added to the cell suspensions. The samples were incubated for 15 min in the dark at room temperature, and immediately subjected to flow cytometric analysis. The The results were interpreted as follows: Lower left quadrant, Annexin V-FITC and PI negative cells; lower right quadrant, Annexin V-FITC positive cells in the early stage of apoptosis; upper left, dead PI positive cells; upper right Annexin V-FITC and PI positive cells in the late stage of apoptosis.

Caspase activity assay. Following incubation with 20 µg/ml 5F for 24 h, the enzymatic activities of caspase-3, caspase-8, and caspase-9 were measured using commercially available caspase-3, -8 and -9 activity assay kits (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China) according to the manufacturer's protocols. The caspase activities were expressed as optical density (OD550/OD_control).

Western blot analysis. Cells were lysed on ice in SDS Lysis Buffer (Beyotime Institute of Biotechnology), and supplemented with protease inhibitors and phosphatase inhibitor (Roche Diagnostics, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). The protein concentration was determined using an Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Immunoblots of 50 µg total protein were probed with primary antibodies overnight as follows: Rabbit anti-human polyclonal cyclin-dependent kinase inhibitor 1A (p21, Cip1) (p21; cat. no. BA0272; dilution, 1:200; Boster Bio-Engineering Ltd., Wuhan, China), rabbit anti-human polyclonal B-cell lymphoma 2 (Bcl-2; cat. no. ZS-492; dilution, 1:300; Zhongshan Golden Bridge Biotechnology, Co., Ltd., Beijing, China), mouse anti-mouse monoclonal Bcl-2-associated X protein (Bax; cat. no. ZS-7480; dilution, 1:300; Zhongshan Golden Bridge Biotechnology, Co., Ltd.), rabbit anti-human polyclonal nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) p65 phosphorylated (p-Ser536 (cat. no. 11014; dilution, 1:500; Signalway Antibody LLC., College Park, MD, USA), rabbit anti-human polyclonal nuclear factor κ light polypeptide gene enhancer in B-cells inhibitor α (IkBα), rabbit anti-human polyclonal survivin (cat. no. sc-10811; dilution, 1:300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and β-actin (cat. no. sc-130656; dilution, 1:500; Santa Cruz Biotechnology, Inc.). Immunoblots were incubated with AP-labeled goat anti-mouse and goat anti-rabbit IgG (H+L) secondary antibodies (cat. nos. A0258 and A0239; dilution, 1:1,000; Beyotime Institute of Biotechnology) for 3 h at room temperature. Protein bands were visualized using chemiluminescent reagents (BeyoECL Plus; Beyotime Institute of Biotechnology, Haimen, China).

Xenograft tumor mouse model. Thirty female BALB/c mice (age, 6 weeks) were obtained from the Guangdong Medical College Experimental Animal Center (Zhanjiang, China). All mice were housed in plastic cages (5 mice/cage) and fed with standard rodent food and tap water. They were housed controlled conditions of humidity (50±10%) and temperature (23±2°C) on a 12:12-h light/dark cycle. Mice were subcutaneously inoculated with 2x10^6 C26 cells in the left armpit region and randomly divided into three groups (n=10) as follows: Saline (control group), 50 mg/kg/day 5F group, and 100 mg/kg/day 5F group. The 5F was dissolved in 0.85% normal saline. The following day, mice were intragastrically administered the specified doses. Their bodyweights were recorded twice each week. On day 14, all mice were sacrificed by cervical dislocation, and the tumor xenografts were removed and weighed. All animal experimental procedures were approved by the Guangdong Medical College Ethics Committee.

Inflammation-associated CRC mouse model. Thirty female ICR mice aged 6 weeks were obtained from Silakejingda Experimental Animal Co., Ltd. (Changsha, China). Twenty mice were administered a single intraperitoneal injection of 1,2-dimethylhydrazine (DMH; 20 mg/kg; Sigma-Aldrich). One week later, the mice were administered a course of 2% (w/v) dextran sodium sulfate (DSS; MP Biochemicals, Solon, OH, USA) in drinking water for one week. The 20 mice were divided into two equal groups (n=10): the model and the 5F (50 mg/kg/day) group. Another 10 mice formed the control group. In the 5F group, 5F was intraperitoneally injected and administered for 35 consecutive days from the first day of the 18th week. In the model group, mice were intraperitoneally administered with normal saline. Assessment of general appearance, bodyweight, food uptake, rectal bleeding and stool consistency was performed every day. All the mice were sacrificed, and the blood and large bowel samples were collected. The number of tumors was counted by two independent observers blinded to the mouse treatment. The colons were fixed in 10% buffered formalin (Beijing Chemical Reagent Factory) for hematoxylin and eosin staining (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China).

Statistical analysis. Data are presented as means ± standard deviation. The differences among the groups were analyzed with one-way analysis of variance using SPSS 13 software (SPSS, Inc., Chicago, IL, USA) and P<0.05 was considered to indicate a statistically significant difference.
Results

5F-induced decrease in cell viability is dose- and time-dependent. To investigate whether 5F prevents the proliferation and growth of CRC cells, C26 cells were treated with different concentrations of 5F for 24, 48 or 72 h, and cell proliferation was detected using a standard MTT assay. 5F treatment led to a time- and dose-dependent decrease in cell viability (Fig. 1). Furthermore, 5F-treated cells exhibited a rounded and granulated morphology, and were detached from the culture wall following a 24-h exposure (Fig. 2).

G2 block is involved in 5F-induced cell cycle arrest. To determine whether the 5F-induced reduction of cell viability was associated with changes in cell cycle distribution in CRC cells, the cell cycle phase in CRC cells incubated with 5F for 24 h were analyzed. 5F treatment arrested CRC cells in the G2 phase of the cell cycle in a dose-dependent manner, as compared with the control group (P<0.01) (Fig. 3).

5F induces cancer cell apoptosis. As 5F treatment induced CRC cell cycle arrest in the G2 phase of the cell cycle, the current study aimed to investigate whether 5F induced CRC cell apoptosis. Data from the present study demonstrate that 5F treatment for 24 h increased the percentage of apoptotic cells (Q2 + Q4; Fig. 4) in CRC cells in a dose-dependent manner, indicating that cell proliferation was inhibited by cell apoptosis (Fig. 4).

Mitochondria pathway is involved in 5F-induced apoptosis. To elucidate whether 5F activates the caspase-dependent apoptosis signaling pathway, the catalytic activity of caspase-3,
Figure 3. Effects of 5F on the cell cycle, as measured by flow cytometric analysis. Cells were treated with (A) 0, (B) 5, (C) 10, (D) 20 and (E) 40 µg/ml 5F for 24 h. (F) Percentage of cells in the G2 phase. *P<0.01 vs. the control. 5F, ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid.

Figure 4. Effects of 5F on cell apoptosis analyzed by flow cytometry. (A) 0, (B) 5, (C) 10, (D) 20 and (E) 40 µg/ml 5F treatment increased the percentages of Annexin-V+/PI- and Annexin-V+/PI+ cells. (F) The data indicated a dose-dependent induction of cell apoptosis at 24 h. Q1, PI positive dead cells; Q2, Annexin V-FITC and PI positive late apoptotic cells; Q3, Annexin V-FITC and PI negative cells living cells; Q4, Annexin V-FITC positive early apoptotic cells. *P<0.01 vs. the control. 5F, ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid; FITC, fluorescein isothiocyanate; PI, propidium iodide.
caspase-8 and caspase-9 was evaluated in CRC cells. The results demonstrate that 5F significantly increased the activity of caspase-3 and caspase-9, however only marginally affected the activity of caspase-8 (Fig. 5), suggesting that 5F-induced apoptosis was mediated by the mitochondrial signaling pathway, but not the death-receptor signaling pathway.

5F alters the expression levels of p21, Bcl-2, Bax, NF-κB, IκBα and survivin proteins. To elucidate the underlying mechanism of 5F-induced apoptosis in C26 cells, the protein levels of p21, Bcl-2, Bax, NF-κB, IκBα and survivin (which are involved in cell cycle progression, growth and apoptosis) were measured. The treatment of C26 cells with 5F resulted in the significant upregulation of p21, Bax, IκBα and downregulation of Bcl-2, NF-κB and survivin (P<0.01) (Fig. 6), suggesting that 5F induces cell apoptosis.

5F inhibits tumor growth in a xenograft tumor mouse model. The xenograft tumor mouse model (induced by C26 cells) was successfully established and it was observed that 5F inhibited the tumor growth. Compared with the mean tumor weight of 1.29±0.22 g in the model group, the mean tumor weights of the 50 and 100 mg/kg/day 5F groups were 0.76±0.19 and 0.45±0.28 g, respectively (Fig. 7). However, there were no significant differences identified in bodyweight, food and water intake or activity between the groups.

Antineoplastic effect of 5F in the inflammation-associated CRC mouse model. General observations were performed on each mouse. Three mice in the model group exhibited rectocele 13, 15 and 16 weeks after commencing the experiment, and 1 mouse in the 5F-treated group exhibited rectoccele 14 weeks after commencing the experiment. The colorectal tumors detected in the CRC model group (induced by DMH/DSS) were identified to be well-differentiated tubular adenocarcinoma and 5F treatment improved the tumor pathology (Fig. 8). The number of tumors was significantly decreased in the 5F group compared with that of the model group (P<0.01), suggesting that 5F significantly inhibits tumorigenesis (Fig. 9A).

5F treatment did not result in major side effects in the inflammation-associated CRC mouse model. The side effects of 5F were investigated in three parts as follows: i) Physiological observations demonstrated no significant weight loss, no reduction in activity levels and no instances of self-torture, self isolation or mortality were observed; ii) liver function tests indicated that the levels of alanine transaminase (ALT) in the model and 5F groups were higher than in the control.
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Group, and highest in the CRC model group; iii) renal function analysis identified no difference in the levels of blood urea nitrogen (BUN) or creatinine (Cr) among all of the groups. Therefore, biochemical parameters and physiological observations indicate that 5F treatment did not result in serious side effects (Fig. 9B).

Discussion

In the present study, 5F has been demonstrated to elicit a growth inhibitory effect on CRC cells, in a xenograft tumor mouse model and in a DMH/DSS-induced tumor mouse model, suggesting that 5F may be administered to treat CRC. Deregulation of the cell cycle and evasion of apoptosis are characteristic of cancer cells (12). Thus, inhibition of cell cycle progression and/or induction of apoptosis is considered to be particularly useful in the treatment of cancer. Previously, 5F has been demonstrated to arrest CNE-2Z, A549 and FRO cells at the G2 phase (3,4,6). Consistent with these studies, the current study demonstrated that 5F induced G2 phase arrest in CRC cells. The p21 protein is an inhibitor of cyclin-dependent kinases (CDKs) and is key in the arrest of cells at the G2/M transition (13,14). Various studies have demonstrated that anticancer therapeutic agents increase the number of cells in the G2/M cell cycle phases, which is accompanied by upregulation of p21 (15-20). The present study demonstrated that 5F increased the expression level of p21 in CRC cells. Apoptosis is important in various normal processes, from fetal development to adult tissue homeostasis, as it eliminates cells that have completed their life cycle, have become ineffective.

Figure 8. Hematoxylin and eosin staining of colon sections in mice from (A and B) the control group treated with saline, (C and D) the model group treated with DMH/DSS, and (E and F) the 5F group treated with DMH/DSS and 5F. 5F, ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid; DMH, 1,2-dimethylhydrazine; DSS, dextran sodium sulfate.

Figure 9. Tumor number and biochemical parameters in the inflammation-associated colon cancer mouse model. (A) The tumor number in 1,2-dimethylhydrazine/dextran sodium sulfate-treated mice (model) and 5F-treated mice. (B) The effect of 5F treatment on liver and renal function of mice. *P<0.05, **P<0.01 vs. the control. Con, control; 5F, ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; Cr, creatinine.
or are harmful to the organism (21). Apoptosis is induced via the extrinsic (death-receptor) or the intrinsic (mitochondrial) signaling pathways. The intrinsic signaling pathway induces the release of cytochrome c from the mitochondria and activates caspase-9. Activated caspase-9 subsequently activates caspase-3, which further cleaves poly (ADP-ribose) polymerase 1 resulting in apoptosis. The extrinsic signaling pathway is triggered by a death ligand binding to a death receptor, which activates caspase-8, further activating caspase-3 directly or activating the intrinsic signaling pathway, which also affects caspase-3 (22-24). The Bcl-2 family regulates the mitochondrial outer membrane permeabilization pore and an elevated ratio of Bax/Bcl-2 is observed in apoptosis of cancer cells (19,25-29). In the present study, 5F markedly increased the percentage of Annexin V/FITC-positive CRC cells, the Bax/Bcl2 ratio and the activity of caspase-3 and caspase-9, however, it did not alter the activity of caspase-8. This suggests that 5F-induced apoptosis in CRC cells was mediated by the mitochondrial signaling pathway and not the death-receptor signaling pathway.

NF-κB is an important mediator of cell cycle progression and cell survival associated with carcinogenesis (30). Under basal conditions, NF-κB is present in the cytoplasm and binds to IκB. Phosphorylation of IκB by IκB kinase results in ubiquitination and degradation of IκB. Subsequently, NF-κB is translocated to the nucleus, where NF-κB regulates the transcription of a number of genes involved in tumorigenesis and cell growth (30,31). NF-κB has been recognized as key in the initiation and progression of CRC (32). Therefore, NF-κB may be a potential therapeutic target in CRC. Results from the present study demonstrated that treatment of CRC cells with 5F inhibited the expression of NF-κB and degradation of IκBα, suggesting that the effects of 5F on NF-κB may be influenced by inhibition of phosphorylation and the subsequent proteolysis of IκBα. Survivin is the smallest member of the family of inhibitors of apoptosis proteins (IAPs) (33). It is overexpressed in the majority of human tumors, and upregulation of survivin has been associated with poor prognosis in patients with tumors, thus, survivin has been proposed as a potential target for anticancer intervention (34,35). In the present study, the effects of 5F on survivin were investigated and it was observed that 5F treatment resulted in a decreased expression level of survivin.

In in vivo experiments, the tumoricidal effect, as well as the side effects of 5F were investigated in two CRC mouse models. Results of the current study indicated that 5F reduced the weight of xenograft tumors and the number of DMH/DSS-induced CRC tumors. Liver and kidney damage are critical and frequent side effects generated by antitumor agents. Therefore, aspartate transaminase (AST), ALT, Cr and BUN were measured to determine the side effects of 5F (36,37). No difference in the levels of AST, Cr and BUN were measured to determine the side effects of 5F. Therefore, aspartate transaminase (AST), ALT, Cr and BUN were measured to determine the side effects of 5F (36,37). No difference in the levels of AST, Cr and BUN were measured to determine the side effects of 5F. Therefore, aspartate transaminase (AST), ALT, Cr and BUN were measured to determine the side effects of 5F.


