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Over-expression of Bcl-2 against Pteris semipinnata L-induced apoptosis of human colon cancer cells via a NF-kappa B—related pathway

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Ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F), an antitumor component, is a chemical compound isolated from Pteris semipinnata L (PsL), a Chinese traditional herb. We examined whether 5F could affect apoptosis in human colon cancer HT-29 cells, and test whether and how the over-expression of Bcl-2 and Bcl-xL could offset the effect of 5F on cell growth. The result demonstrated that 5F significantly induced apoptosis of HT-29, as shown by MTT assay and DNA fragmentation measurement. Treatment of HT-29 with 5F increased both p38 and iNOS levels, suggesting these two molecules may contribute to the apoptotic effect of 5F. Over-expression of Bcl-2 or Bcl-xL attenuated the increase of p38 and iNOS induced by 5F. The cells with Bcl-2 or Bcl-xL over-expression showed an elevation of nuclear factor kappa B (NF-kappa B) activity, accompanying a significant reduction of 5F-induced apoptosis. Furthermore, inhibition of NF-kappa B by IκBαSR, which is a powerful inhibitor of NF-kappa B, restored the ability of 5F to induce apoptosis in the cells transfected with Bcl-2. These data strongly indicated that the apoptotic effect of 5F on HT-29 was closely associated with the activity of NF-kappa B, which was up-regulated by Bcl-2 and Bcl-xL. In conclusion, 5F induced apoptosis in HT-29 cells and this apoptotic effect was associated with the high level of p38 and iNOS expression. The apoptotic effect of 5F could be significantly offset by over-expression of either Bcl-2 or Bcl-xL. Bcl-2, and to the less extent, Bcl-xL, were able to increase the activity of NF-kappa B, which was a known anti-apoptotic molecule in human colon cancer cells.

Keywords: apoptosis; Bcl-2; colon cancer; nuclear factor kappa B; Pteris semipinnata.

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Introduction

Pteris semipinnata L (PsL) is a Chinese traditional herb, which has been used to treat variety of inflammatory conditions such as hepatitis, enteritis and snake bite.[1] Recently, several chemical compounds have been identified from the ethanolic extract of the PsL. Some of these compounds are able to inhibit the growth of tumor cells, including gastric adenocarcinoma cells (MGC-803), lung adenocarcinoma cells (SPC-A-1), human promyelocytic leukemia cells (HL-60), nasopharyngeal carcinoma cells (CNE-2Z), and liver adenocarcinoma cells (BEL-7402, HepG2).[2,3] Further studies reveal that PsL-induced cell arrest is associated with reduced DNA topoisomerase,[1] abnormal activation of mitogen activated protein kinase,[4] decreased Bcl-2 expression and high levels of Bax, c-Foc and c-Jun.[5] However, the effect of PsL on human colon cancer cells has not yet been investigated.

Nuclear factor kappa B (NF-kappa B), a redox-sensitive transcription factor, is regulated by various apoptotic stimuli or inhibitors. A number of reports have shown that NF-kappa B is inhibited by apoptosis-inducing agents in human colon cancer cells.[6–8] Like Bcl-2 and Bcl-xL, NF-kappa B itself may serve as a pro-survival agent in various circumstances.[9] The activation of NF-kappa B is known to induce the expression of Bcl-2 and Bcl-xL.[10–14] Inducible loss of NF-kappa B activity is associated with the down-regulation of anti-apoptotic Bcl-2 family members and the occurrence of apoptosis. Furthermore, the promoter regions of both Bcl-2 and Bcl-xL genes contain binding sites for NF-kappa B.[13–15] On the other hand, Bcl-2 can also stimulate or restore NF-kappa B activity.[16,17]

In the present experiment, we examined whether ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F), an effective antitumor component from PsL,[1–5] could affect apoptosis in human colon cancer cells. In order to test whether and how the over-expression of Bcl-2 and Bcl-xL

offsets the effect of PsL on cell growth, we transfected human colon cancer cells with either Bcl-2 or Bcl-xL gene.

Materials and methods

Cell culture

A human colorectal cancer cell line, HT-29, was purchased from American Type Culture Collection (Rockville, Maryland), and cultured in RPMI 1640 medium (GIBCO BRL, Grand Island, New York) containing 10% heat-inactivated fetal bovine serum and antibiotics. Cells were treated with either 5F or 7-hydroxystaurosporine (UCN-01) at concentrations indicated. 5F was prepared as previous description.[2–4] UCN-01 was kindly provided by the National Cancer Institute (Rockville, MD).

Quantitation of cell death and apoptosis

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to quantify cell death/viability. Cell death was also determined by DNA fragmentation assay kit (Roche Molecular Biochemicals, Mannheim, Germany), which measures apoptotic cell death by detection of BrdU-labled DNA fragments in the cytoplasm of affected cells. Briefly, DNA fragments in the cytoplasm were labeled with BrdU. After the BrdU-labeled DNA fragments were then isolated from the cells using the solutions provided by the kit, the BrdU-labeled DNA fragments were then detected using an enzyme-linked immunosorbent assay (ELISA) method. The entire assay was performed according to the manufacturer's instruction. Appearance of DNA fragments has been considered as a hallmark of apoptosis. Apoptotic cells were also evaluated by TUNEL assay using an APO-Direct Flow Cytometry Kit from Chemicon (Temecula, CA). The TUNEL assay was performed according to the kit instruction from Chemicon.

DNA transfection

Stable transfection was performed using cationic lipid reagent, LipofectAMINE (Life Technologies, Rockville, Maryland). The experiment was carried out according to the manufacturer's instruction. HT-29 cells (80–85% confluence) were transfected with 500 ng of plasmid DNA in serum-free conditions. After the cells were incubated for 6 hours in serum-free medium containing DNA and LipofectAMINE, an equal volume of growth medium containing 20% serum was then added without removing the transfection mixture. The supernatant was changed with complete medium at 24 hours following the start of transfection. At 72 hours after transfection, the cells were passaged into the selective medium containing geneticin selective antibiotic, G418. The concentration of G418 for the selection was previously determined by dose-response assay. Positive stably-transfected cells were selected by G418 (0.4 mg/ml) after 2 months.

Western blot analysis

Cell samples were homogenized with ice-cold PBS and then lysed in a solution containing 8 M urea, 0.1 M $Na_2H_2PO_4$ and 0.01 M Tris-HCl. Supernatants were obtained after centrifugation at 10, 000 $\times$ g. Proteins were separated on 10% SDS-polyacrylamide gels. Proteins were then electrophoretically transferred from the gel onto nitrocellulose membranes and the membranes were blocked for 1 hour in PBS-Tween buffer containing 5% dry milk powder (fat free) at room temperature. The membranes were then incubated with a primary antibody for 1 hour. After washing, the membranes were incubated with a secondary antibody, IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, California). Finally, they were treated with the reagents in the chemiluminescent detection kit (ECL system, Amersham Pharmacia Biotech, Piscataway, New Jersey) according to the manufacturer's instructions. Anti-human actin antibody (Santa Cruz Biotechnology, Santa Cruz, California) was used to detect human actin, which was used as a control for equal loading. Anti-human antibodies: Bcl-2, Bcl-xL, Bid, p65, cyclooxygenase-2 (COX-2), inducible nitric oxide (iNOS) and p38 mitogen-activated protein kinase (p38 MAPK) were obtained from Santa Cruz Biotechnology (Santa Cruz, California). The densities of the protein bands corresponding to the size were determined with a GSP-700 scanner with Quantity One image software (Hercules, CA).

Nuclear protein extraction and NF-kappa B activity assay

Nuclear protein was isolated according the procedure described.[18] Briefly, HT-29 cells were harvested, washed in PBS, and collected by centrifugation. The cell pellet was resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/0.05% (v/v) Triton X-100 and lysed with 20 strokes in a homogenizer. The homogenate was centrifuged at 10, 000 $\times$ g for 15 minutes at 4°C. The pellet was obtained. The nuclei pellet volume was estimated and the pellet was resuspended in an equal volume of 10 mM Tris-HCl (pH 7.4)/5 mM $MgCl_2$, followed by the addition of 1 nuclear pellet volume of 1M NaCl/10 mM Tris-HCl (pH 7.4)/4 mM $MgCl_2$. The lysing nucleus was left on ice for 30 minutes and then centrifuged at 10, 000 $\times$ g for 15 minutes at 4°C. The supernatant (nuclear extract)

was removed and 80% glycerol was added so that the final glycerol concentration was 20% (v/v). The concentration of the nuclear protein was determined. NF-kappa B activity was measured by an enzyme immunoassay kit from Oxford Biomedical Research (Oxford, MI), which employed an oligonucleotide containing the DNA binding NF-kappa B consensus sequence. If there is any NF-kappa B presented in the sample, it will specifically bind to the oligonucleotide coated on the plate and the DNA-bound NF-kappa B can be selectively recognized by the antibody to NF-kappa B subunit, p50 or p105.

Inhibition of NF-kappa B

A recombinant replication-deficient adenovirus, Ad5IkB, contains an IkB construct in which serines 32 and 36 are mutated to alanines, driven by the cytomegalovirus promoter-enhancer. This mutant IkB cannot be phosphorylated, and therefore irreversibly binds to NF-kappa B, preventing its activation.[19,20] Ad5IkB (a generous gift from Dr. D Brenner) was used as previously described.[20] The adenovirus, Ad5LacZ, which contains the *Escherichia coli* β-galactosidase gene, was used as a control. Both viruses were grown in 293 cells and purified as the previous description.[20] For adenovirus infection, subconfluent cells ($\sim$80%) were infected with virus in serum-free medium at multiplicity of infection (MOI) of 10–500 for 12 hours. The free virus was then washed off and fresh media containing serum was added to the cells. The cells were cultured for another 12 hours before experiments.

Statistical analysis

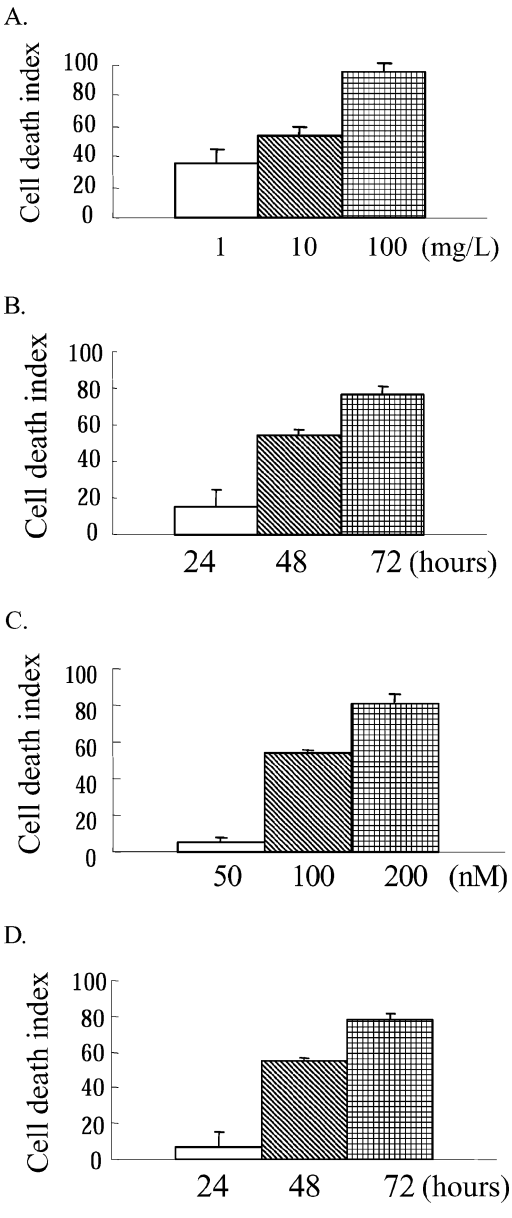
All values were expressed as mean $\pm$ standard error. Statistical comparisons were analyzed by the Student's t test using InStat software (GraphPad Software, San Diego, California). A P-value of less than 0.05 was taken as statistically significant.

Results

5F induced cell death in dose- and time-dependent manners

5F induced HT-29 cell death in a dose-dependent manner between the concentrations of 1 and 100 mg/L (Figure 1A). At the concentration of 100 mg/L, over 95% of the cells were dead after 48-hour treatment. The effect of 5F was also found in a time-dependent fashion (Figure 1B). Compared with 24-hour treatment with 10 mg/L of 5F, the cell death was gradually increased to about 3.5 folds and 5.1 folds after 48-hour and 72-hour treatments with 5F respectively. Both the dose- and the time-dependent manners were similar to the results obtained when the cells were treated with UCN-01 (Figures 1C and D).

Figure 1. Cell death induced by 5F and UCN-01. After HT-29 cells were treated by 1-100 mg/L of 5F or 50–200 nM of UCN-01 for 48 hours (A and C), the cell was harvested and the MTT assay was performed to measure cell death. For the time-course study, the cell was treated with either 10 mg/L of 5F or 100 nM of UCN-01 for 24, 48 and 72 hours (B and D). At the end of the time point, the cell death was measured by MTT assay. Control cells were treated with vehicle only. The result of MTT was expressed as Cell Death Index, which was calculated by the following formula: [1-absorbance(test well-background)/absorbance (control-background)] $\times$ 100%. Each experiment was repeated at least 3 times.



5F upregulated the expression of p38 MAPK and iNOS but downregulated the levels of Bcl-2 and Bcl-xL

To understand how 5F regulates cell death, we measured several cell death-related molecules in HT-29 cells treated with 5F. By Western blot analysis, we found that the expression of p38 MAPK and iNOS was much higher in the cells treated with 5F than those treated with vehicle, whereas the levels of p65, Bcl-2 and Bcl-xL were lower (Figure 2). The expression of COX-2 and other Bcl-2 family members such as Bid and Bcl-xs was not different between the treatment and the control (data not shown). The similar expression patterns of these molecules analyzed were also observed in the cells treated with UCN-01 (Figure 2). The alteration of p38 MAPK, iNOS, Bcl-2 and Bcl-xL could be occur as early 24 hours (data not shown), which predated the activation of caspase-3. Time course study showed that a slight increase in 32 kDa caspase-3 at 36 hours and the active caspase-3 (20 kDa) did not appear until 48 hours after 5F treatment (Figure 3).

Over-expression of Bcl-2 and Bcl-xL protected cell death induced by 5F

To investigate how the over-expression of Bcl-2 and Bcl-xL affects the cell death in HT-29 induced by 5F, we transfected HT-29 cells with Bcl-2, Bcl-xL, or pcDNA3.1 plasmids and the cells were named as HT-29/Bcl-2, HT-29/Bcl-xL and HT-29/mock cells respectively. Positive clones were selected by G418 and the over-expression of Bcl-2 or Bcl-xL was confirmed using Western blot (Figure 4). In order to clarify whether the over-expression

Figure 2. Effect of 5F and UCN-01 on the protein levels of p38 MAPK, iNOS, Bcl-2 and Bcl-xL. HT-29 cells were treated with 10 mg/L of 5F or 100 nM of UNC-01 for 48 hours and then cell lysates were obtained for Western blot analysis of p38 MAPK (38 KDa), iNOS (130 KDa), p65 (65 KDa); Bcl-2 (28 KDa) and Bcl-xL (32 KDa). Actin protein (43 KDa), which is constitutively expressed, was used as a control. The densities of the protein bands corresponding to the size were determined and the relative amount of the target protein was shown as Expression Index, which was calculated by the formula: the density of the control (actin) band/ the density of the target band.

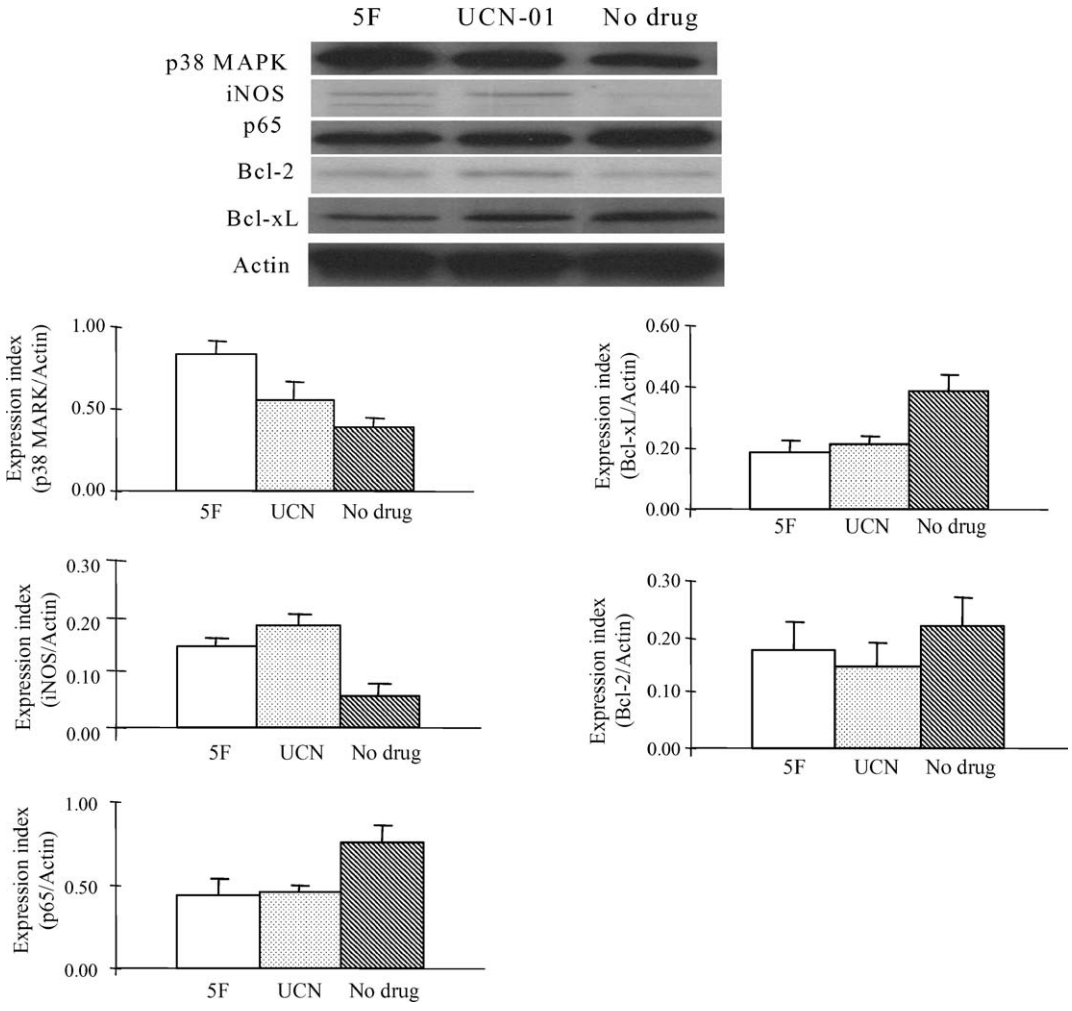


Figure 3. Time-course study of caspase-3 expression. HT-29 cells were treated with 10 mg/L of 5L and cell lysates were obtained at 0 (without 5L treatment), 24, 36, 48 and 60 hours for protein analysis. Detection of caspase-3 protein was performed using the ECL Western blotting detection system.

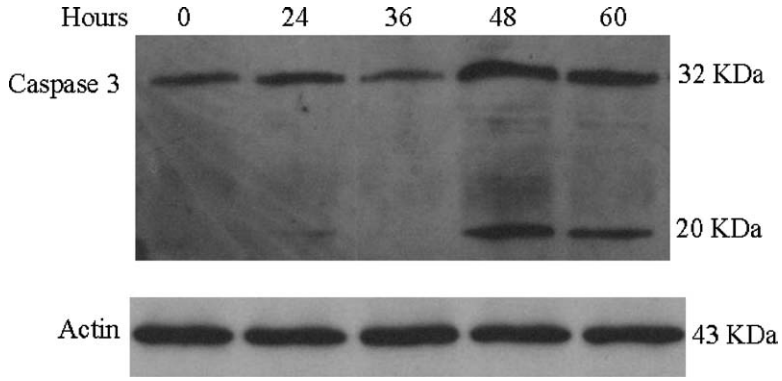
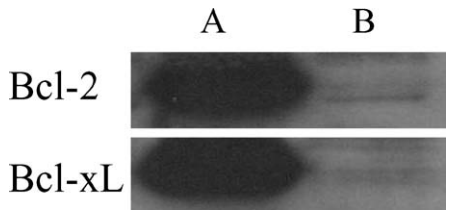


Figure 4. Over-expression of Bcl-2 and Bcl-xL in HT-29. After stable transfection, HT-29/Bcl-2 and HT-29/Bcl-xL cells were established. Cell lysates were isolated from the cells and Bcl-2 (28 KDa) and Bcl-xL (32 KDa) levels were determined by Western blot analysis. A: The cells transfected with Bcl-2 or Bcl-xL genes; B: The cells transfected with an empty vector.



of Bcl-2 or Bcl-xL in HT-29 cells blocks the chemical induction of apoptosis, HT-29/Bcl-2, HT-29/Bcl-xL and HT-29/mock cells were treated with a known cell death inducer, UCN-01 (Figure 5). Following treatment, cell death was measured by MTT assay. The result showed that HT-29/Bcl-2 and HT-29/Bcl-xL cells had a significantly higher percentage of living cells compared to HT-29/mock cells (Figure 5A), indicating that the HT-29/Bcl-2 and HT-29/Bcl-xL transfectants were functional. These cells were then treated with 10 mg/L of 5F (Figure 5B). While HT-29/mock cells displayed marked death in response to 5F treatment, HT-29/Bcl-2 and HT-29/Bcl-xL cells showed approximately 95% and 90% protection from 5F-induced death respectively. The cell death induced by either 5F or UCN-01 was mainly caused by apoptosis, as the similar result was obtained by DNA fragmentation assay (Figures 5C and D). The occurrence of DNA fragment is a typical Marker for apoptosis. Apoptosis was further confirmed by TUNEL assay (APO-Direct Flow Cytometry Kit, Chemicon, Temecula, CA). After 5F treatment, HT-29/Bcl-2 and HT-29/Bcl-xL cells exhibited only 1.51% and 0.25% of apoptotic cells while the control cells (without treatment or vector transfection only) showed average 82.46% of apoptotic cell death, suggesting that 5F could offer 98–99% protection against the cytotoxic effect of 5F.

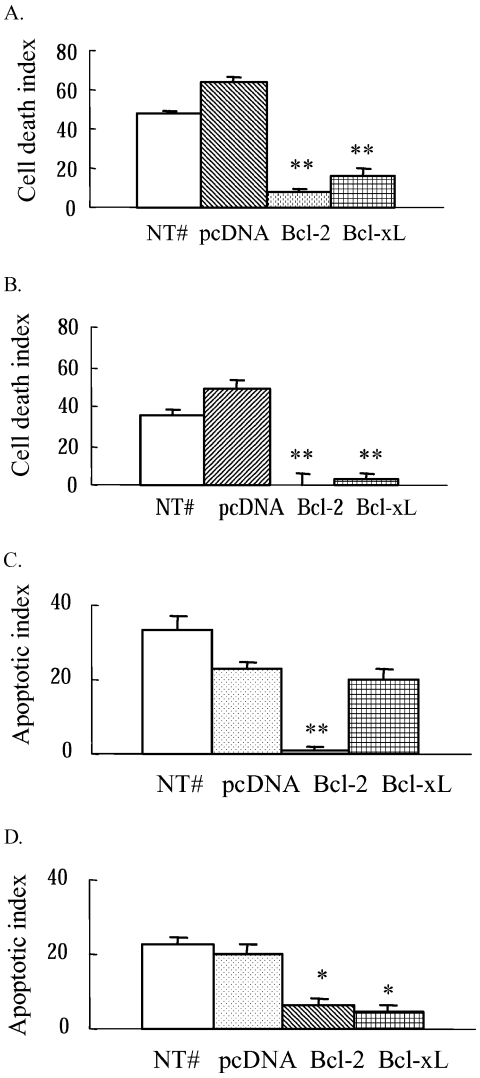
Over-expression of Bcl-2 and Bcl-xL increased NF-kappa B activity

NF-kappa B activity is known to be closely associated with Bcl-2 and Bcl-xL expression.[10–14,16,17] In order to determine how the over-expression of Bcl-2 and Bcl-xL affects the NF-kappa B activity in HT-29 cells treated either 5F or UCN-01, the cells transfected with either Bcl-2 or Bcl-xL were incubated with 10 mg/L of 5F, 100 nM of UCN-01 or vehicles. The nuclear protein was isolated after incubation. The cells transfected with an empty pcDNA3.1 vector and the cells without any transfection were used as controls. NF-kappa B activity was significantly higher in the cells with Bcl-2 or Bcl-xL (Figure 6). It appeared that Bcl-2 was more effective in raising NF-kappa B activity than Bcl-xL.

Inhibition of NF-kappa B offsetted the protective effect offered by Bcl-2 and Bcl-xL

Since the over-expression of Bcl-2 and Bcl-xL increased the activity of NF-kappa B and protected the cells from apoptosis induced by 5F and UCN-01, it would be interesting to know whether the inhibition of NF-kappa B activity could diminish the protective effect offered by Bcl-2 and Bcl-xL. A super-repressor of NF-kappa B activity (Ad5IkB), which is a mutated non-degradable IkBα resistant to phosphorylation and degradation, was used to inhibit the activity of NF-Kappa B. Previous experiments have demonstrated that this super-repressor was capable of inhibiting the activity of NF-kappa B in HT-29 cells. HT-29/Bcl-2 cells regained their sensitivity to apoptosis induced by 5F after being treated by Ad5IkB (Figure 7A).

Figure 5. Protection of cell death induced by UCN-01 and 5F with Bcl-2 or Bcl-xL. HT-29 cells with extraneous genes (Bcl-2 or Bcl-xL) or without were incubated with 10 mg/L of 5F (B and D), 100 nM of UCN-01 (A and C) or vehicles for 48 hours. The cell death was measured by the MTT assay (A and B). The result of MTT was expressed as Cell Death Index (see Figure 1 for details). The cell apoptosis was determined by DNA fragmentation detection assay (C and D). DNA fragments from damaged cells were released into the culture supernatant and labeled by BrdU. The BrdU-labled DNA was quantified using a monoclonal antibody against BrdU. Each point represents a mean of three independent experiments with triplicate wells. The result of DNA fragmentation detection assay was expressed as Apoptotic Index, which was calculated by the following formula: [1-absorbance(test well-background)/absorbance (control-background)] × 100%. #NT: No (UNC-01 or 5F) treatment. $^{**}p < 0.01$,$^{*}p < 0.05$ compared with the controls.



However, a significant portion of HT-29/Bcl-xL cells was not protected after treated by Ad5IkB (Figure 7A), suggesting that Bcl-xL could still function, to a certain degree, to protect the cells from death when the activity of

Figure 6. NF-kappa B activity in the cells with Bcl-2 and Bcl-xL over-expression. HT-29 cell culture was the same as that described in Figure 4 and cells were with 10 mg/L of 5F (B), 100 nM of UCN-01 (A) or vehicles for 48 hours. After culture, the nuclear protein was isolated for the determination of NF-kappa B activity, which was measured by a commercially-available ELISA kit. Each point represents a mean of three independent experiments with triplicate wells. #NT: No (UNC-01 or 5F) treatment. $^{**}p < 0.01$,$^{*}p < 0.05$ compared with the controls.

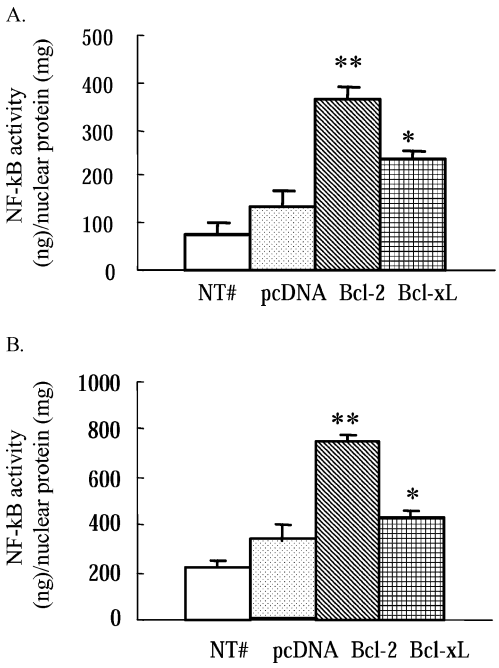
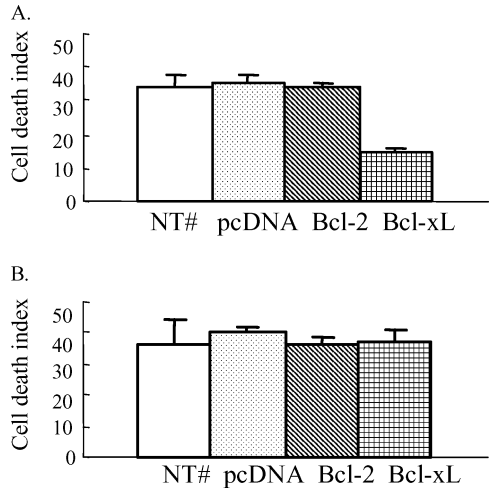


Figure 7. Attenuation of Bcl-2 and Bcl-xL protection by the inhibition of NF-kappa B activity. The cells were infected with either Ad5IkB or Ad5LacZ, as described in Methods. After the infection, the cells were stimulated with 5 F 10 mg/L of 5F (A), 100 nM of UNC-01 (B) or vehicle alone for 48 hours. The cell death was measured by the MTT assay. The result of MTT was expressed as Cell Death Index (see Figure 1 for details). #NT: No (UNC-01 or 5F) treatment.



NF-kappa B was inhibited. Somewhat different from 5F, cell death induced by UCN-01 in both HT-29/Bcl-2 and HT-29/Bcl-xL cells were prevented when the cells were treated by Ad5IkB (Figure 7B), indicating that both Bcl-2 and Bcl-xL lost their anti-apoptotic effects when the activity of NF-kappa B was suppressed.

Discussion

Although 5F, a chemical component isolated from PsL, has been known to inhibit the growth of several types of tumor cells,[1–5] the mechanism is not yet clear and its effect on human colon cancer cells has not yet been studied before. In the present study, we demonstrated that 5F was able to induce cell death via an apoptotic pathway and in a similar manner to UCN-01. UCN-01 was originally identified as a more specific inhibitor of protein kinase C (PKC) than the parent compound staurosporine.[21,22] However, the inhibition of PKC is unlike to be responsible for its effect on the cell growth arrest. UCN-01 showed the growth-inhibitory effect against malignant cells, as demonstrated in the present and others' studies.[23,24] UCN-01 is currently undergoing clinical evaluation as an antitumor drug in the United State and Japan. 5F at the concentration of 10 mg/L seems to be able to achieve the same efficiency of UCN-01 at 100 nM, as both drugs at these concentrations induced a similar number of cell death and also they were able to generate very similar time-course curves. Therefore, by comparing 5F with a well-known cell growth inhibitor, such as UCN-01, it will help us to evaluate whether 5F can be considered to be an anti-cancer agent.

In the present study, we found that a number of molecules were altered at protein levels in human colon cancer cells treated by 5F. Both p38 MAPK and iNOS were increased by either 5F or UCN-01 treatment. It is widely accepted that iNOS is a main enzyme responsible for a large amount of nitric oxide (NO) production. NO is apoptosis-inducer in a variety of human cells and has been a target for novel therapy in a number of malignant cells. For example, in human gastric cancer, NO contributes to the tumouricidal activity of 5-fluorouracil.[25] In human colon cancer cells, NO generated by interferon-gamma and tumor necrosis factor alpha induces cell death.[26] NO donors, such as glyceryl trinitrate, are known to induce apoptosis via a pathway related to activating caspase cascade, increasing the expression of Fas and decreasing the expression of several inhibitors of apoptosis (IAPs).[27] The production of NO is closely associated with p38 MAPK. p38 MAPK is involved in the generation of NO, at least, at two points. First, the p38 MAPK pathway can enhance NO production by increasing the intracellular concentration of the substrate for NO synthetase via stimulation of arginine transporter activity.[28] Second, p38 MAPK activity is required for the increased gene expression of iNOS.[29] In addition, activation of p38 MAPK is also involved in the NO-induced apoptosis by relaying NO signal to its down stream apoptotic molecules including p53, cytochrome c and caspases.[30,31] The increased p38 MAPK and NO levels are reported to participate cell death induced by UCN-01,[23,32] which is in line with the current findings. Therefore, the result of increased iNOS and p38 MAPK is thought to contribute to the cell death induced by 5F.

NF-kappa B has recently emerged as a major player in a variety of human cancers mainly because of its ability to protect transformed cells from apoptosis. There is increasing evidence to support a role for NF-kappa B in tumorigenesis of colon cancer and to document that the inhibition of NF-kappa B suppresses the growth of colon cancer cells via an apoptotic pathway,[6,7,33,34] which is impaired during colon cancer development.[6,35] Dietary flavone dramatically reduced NF-kappa B activity, together with COX-2 levels.[34] The reduction was displayed a high selectivity for the induction of apoptosis and of growth inhibition. The mechanism of 5F on human colon cancer cells may be different from flavone, as it didn't change the COX-2 expression though it did reduce the activity of NF-Kappa B. We found that accompanying the decreased NF-kappa B activity by 5F was the reduction of two anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-xL. The finding is not surprising, as both the promoter regions of Bcl-2 and Bcl-xL genes contain binding sites for NF-kappa B and thus their expression is controlled by the activity of NF-kappa B.[13–15] It has been reported that NF-kappa B activity is negatively associated with p38 MAPK. In human melanoma cells, p38 MAPK suppresses NF-kappa B activity, which sensitizes melanoma cells to UV-, ribotoxic and radiomimetic chemicals-induced apoptosis.[36] Although 5F also reduces NF-kappa B activity and elevates the level of p38 in human colon cancer cells, the pathway linked these two molecules needs further study.

The decreased NF-kappa B activity, the reduction of Bcl-2 and Bcl-xL levels and the increased p38 and iNOS expression in the colon cancer cells treated with 5F were in agreement with a significantly increase in the number of cell death and the amount of DNA fragmentation, a classic indicator of apoptosis. In order to further support our findings, we transfected the cells with Bcl-2 or Bcl-xL genes and tested how these transfectants behaved in response to 5F stimulation. After transfection with either Bcl-2 or Bcl-xL, the cells showed a significant resistance to the 5F treatment and the percentage of cell death was markedly different from those without Bcl-2 or Bcl-xL transfection. And also the activity of NF-kappa B was significantly higher in the cells with Bcl-2 or Bcl-xL genes. The current result may support the notion proposed by the previous reports that Bcl-2 overexpression preserves

or restores NF-kappa B and thus inhibits apoptosis.[16,37] Furthermore, using a super-repressor of NF-kappa B, the cells with Bcl-2 transfection re-gained their sensitivity to 5F treatment. However, the cells with Bcl-xL over-expression failed to have full recovery of their sensitivity to the drug stimulation. Therefore, it appears that the protective mechanism of Bcl-2 and Bcl-xL is different, to some extent, in colon cancer cells in response to 5F stimulation, with the former being much more NF-kappa B-dependent than the latter. This is also one aspect that is different between 5F and UCN-01, whose treatment led to the similar level of NF-kappa B-dependent inhibition in the cells with the over-expression of either Bcl-2 or Bcl-xL. The mechanism accounting for the different levels of NF-kappa B-dependence in the cells with Bcl-2 and Bcl-xL over-expression is worth further investigating.

Though both 5F and UCN-01 can offer anti-cancer effects probably via a similar mechanism, it is not clear what the advantage is of using 5F in comparison to UCN-01. However, *in vivo* study of UCN-1 has shown that UCN-01 can generate a number of side- or toxicological effects such as body weigh lose, leucopenia, bone marrow depletion and lymphoid tissue atrophy.[38] Traditional Chinese herb medicine is usually tranquil. Nevertheless, further studies need to evaluate 5F as an anti-cancer agent and also its side effects.

Conclusion

5F induced apoptosis in HT-29 cells and this apoptotic effect was associated with the high level of p38 and iNOS expression. The apoptotic effect of 5F could be significantly offset by over-expression of either Bcl-2 or Bcl-xL. Bcl-2, and to the less extent, Bcl-xL, were able to increase the activity of NF-kappa B, which was a known anti-apoptotic molecule in human colon cancer cells. Inhibition of NF-kappa B can restore the ability of 5F to induce apoptosis. Therefore, apoptotic effect of 5F on colon cancer cells is closely related to the activity of NF-kappa B.

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