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A rice bran polyphenol, cycloartenyl ferulate, elicits apoptosis in human colorectal adenocarcinoma SW480 and sensitizes metastatic SW620 cells to TRAIL-induced apoptosis

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ABSTRACT

High intake of whole grain food has been suggested as an important factor for reducing the risk of colon cancer, owing to the abundance of indigestible fibers. Our findings demonstrated that, among various rice bran phenolic compounds tested, cycloartenyl ferulate (CF) showed the most prominent in vitro growth inhibition on human colorectal adenocarcinoma SW480, but had low toxicity on normal colon CCD-18-Co cells. The anticancer activity of CF was further illustrated by its ability to induce significant regression of SW480 xenograft in nude mice. CF elevated the death receptors DR4 and DR5 and triggered both the death receptor and the mitochondrial apoptosis pathways. Depletion of anti-apoptotic Bcl-2 and up-regulation of pro-apoptotic Bak were observed, accompanied by dissipation of the mitochondrial membrane potential and release of cyto *c* and SMAC/DIABLO from mitochondria into the cytosol. Bid was found to be cleaved by caspase-8, so that the death receptor pathway might be exaggerated by the mitochondrial pathway. Strikingly, we showed for the first time that CF also sensitized the metastatic and resistant colon cancer SW620 to TRAIL-induced apoptosis and the mechanisms involved at least enhanced activation of caspase-8 and -3. This study provides a clear evidence that the health-beneficial properties of whole grain consumption are not only limited by the presence of dietary fibers but also other molecules that can either act as a chemopreventive agent to directly induce tumor regression or as a sensitizer to enhance TRAIL-induced apoptosis in metastatic cancer cells.

1. Introduction

Colorectal cancer (CRC) is the most common form of lower gastrointestinal cancer and is a leading cause of morbidity and mortality in both men and women in the Western world. About 75% of patients with CRC are attributed to sporadic disease, with no apparent evidence of having inherited the disorder. The remaining 25% of patients have a family history of CRC associated with a genetic contribution, common exposures among family members, or a combination of both. CRC arises through a gradual series of well-characterized histopathological changes, also known as the adenoma–carcinoma sequence. 5-Fluorouracil was first introduced for the cure of CRC, but its response rate is only 15% in metastatic CRC [1]. Subsequently, the addition of associated adjuvant agents is used in the first-line chemotherapy, though development of chemoresistance in some patients is still a challenge. Recently, a humanized anti-VEGF monoclonal antibody (bevacizumab; Avastin) has been approved by the FDA in combination with chemotherapy against metastatic CRC, though adverse side effects have been observed [2,3]. In addition to developing therapeutic regimens against CRC, recent progress in understanding carcinogenesis at molecular level increases the likelihood that anticancer will increasingly rely on chemoprevention, which can delay, retard or reverse the carcinogenesis through a long-term use of a variety of oral agents. Although FDA approved the use of celecoxib as a chemopreventive agent against colon carcinogenesis, in many but not all clinical studies, it was found to associate with higher risks of myocardial infarction and stroke [4]. Consequently, developing chemopreventive and/or therapeutic regimens to combat CRC without significant side effects arouses scientists' great interest.

Higher frequency of whole grain food intake has been suggested as an indicator of reduced risk of several digestive tract neoplasms, including stomach, colon and gall bladder [5]. Plenty of indigestible fibers, as well as several nutrients, such as antioxidants, indoles and phenolic compounds present in whole grain has been regarded

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Abbreviations: CF, cycloartenyl ferulate; CRC, colorectal cancer; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; FADD, Fas-associated death domain; cyto *c*, cytochrome *c*; SMAC, second mitochondrial derived activator of apoptosis; DIABLO, direct IAP binding protein with low PI; AIF, apoptosis inducing factor; IAP, inhibitors of apoptosis protein; DISC, death-inducing signaling complex.

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as the beneficial part of its consumption [6]. Rice, *Oryza sativa*, is the staple dietary constituent of over half of the population of the world. Rice bran, which is a component of raw rice after removal of starchy endosperm in milling process, has been shown to be a rich source of some health-beneficial compounds for preventing cancer, hyperlipidaemia, fatty liver, hypercalciuria, kidney stones, and heart disease [7]. Since several food polyphenols have been shown to have chemopreventive properties by reducing the incidence of many types of cancers, especially colon epithelia [8], the current study was performed to examine the growth-inhibitory effect of various rice bran phenolic compounds on a panel of CRC cells, including SW480 (stage B), SW620 (stage C) and Colo-201 (stage D) with increasing metastatic potential according to the Dukes' classification system. Among the phenolic compounds, cycloartenyl ferulate (CF) was chosen for further investigation for the mechanisms responsible for the growth inhibition in SW480 cells, which are in the early stages of cancer development. CF is one of the typical triterpene alcohols present in and unique to rice bran oil (Fig. 1). CF is a major component of gamma-oryzanol, which has been approved as pharmaceutical in Japan. In the two-stage carcinogenesis model in mouse skin, CF inhibited tumor promotion, indicating a role in cancer chemoprevention [9]. Recently, CF has been demonstrated to possess potentially anti-inflammatory and anti-oxidative activities in RAW 264.7 macrophage via NF-κB inactivation [10]. Nonetheless, study of the anticancer activities of CF is scarce.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a promising candidate for cancer therapeutics due to its ability to induce apoptosis selectively in cancer cells [11]. TRAIL elicits apoptosis by binding to death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2). On the other hand, it also binds to the decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) lacking the cytoplasmic tails that cannot transmit death signals. Interaction between TRAIL and death receptors recruits the adaptor protein Fas-associated death domain (FADD) and subsequently causes activation of initiator caspase-8 and -10. In type I cells, initiator caspases cleave and activate effector caspases leading to apoptosis. In type II cells, the apoptotic signal is amplified by participation of mitochondria. Initiator caspases cleave and activate a Bcl-2 family member Bid, which eventually induces mitochondrial dysfunction and caspase-9 activation. Soluble recombinant TRAIL is in a phase I clinical trial for the treatment of solid tumors [12]. However, some common cancers, especially those with malignant phenotypes, have been identified with TRAIL resistance and thus evaded apoptosis. Therefore, it is important to develop agents that are able to sensitize the cancer cells to TRAIL, or to recover TRAIL sensitivity. We show for the first time that CF not only induces growth inhibition in SW480 cells, which are at early stages of colorectal carcinogenesis, but also sensitizes the metastatic and resistant SW620 cells to TRAIL treatment via at least enhanced activation of the extrinsic pathway of apoptosis.

2. Materials and methods

2.1. Materials

Rice bran phenolic compounds caffeic acid, ferulic acid, methoxycinnamic acid, *p*-coumaric acid, protocatechuic acid, sinapic acid and vanillic acid were purchased from Sigma, St. Louis, MO; tricin from Syncom, Groningen, The Netherlands; and gamma-oryzanol and cycloartenyl ferulate from Wako, Osaka, Japan. β-Actin was purchased from Sigma; TRAIL and DR4 antibodies from BD Pharmingen, San Diego, CA; and antibodies for AIF, bad, bak, bax, bcl-2, bcl-xL, bid, bik, bim, bmf, bok, caspase-3, -6, -7, -8, -9, -10, cytochrome *c*, DR5, mcl-1, poly(ADP-ribose) polymerase (PARP), puma and SMAC/DIABLO were purchased from Cell Signaling Technology, Danvers, MA. Caspase inhibitors, z-VAD-fmk and z-IETD-fmk, and human recombinant TRAIL were purchased from Calbiochem, La Jolla, CA.

2.2. Cell culture

Human colorectal adenocarcinoma cell lines, including SW480, SW620 and Colo-201, and a normal colon cell line, CCD-18-Co, were purchased from American Type Culture Collection (Rockville, MD). SW480 and SW620 cells were grown in Leibovitz L-15 medium (Gibco, Rockville, MD), while Colo-201 cells were grown in RPMI 1640 medium (Sigma). The normal cell line was grown in EMEM medium (Gibco). All media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma), and RPMI-1640 was additionally supplemented with 0.2% sodium bicarbonate (Sigma) and 20 mM sodium pyruvate (Gibco). Colo-201 and CCD-18-Co cells were maintained under a fully humidified atmosphere with 5% CO_2, whereas SW480 and SW620 cells were cultured without CO_2 at 37 °C. All cultures were routinely passaged twice a week.

2.3. Determination of cell growth

Growth inhibition of the rice bran phenolic compounds was determined on a panel of human CRC cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Different cancer cells (1×10^4) were seeded in 96-well plates and allowed to grow for 24 h for acclimatization. The cells were then incubated with 0 or 200 μM rice bran phenolic compounds for 72 h. All samples were dissolved in DMSO with

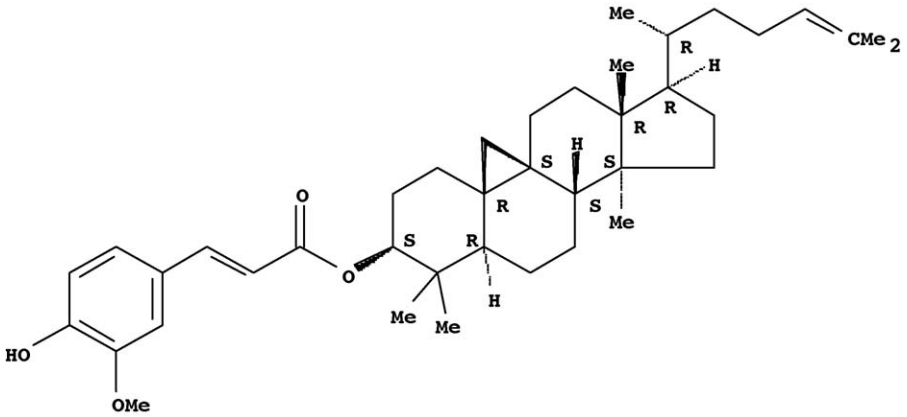


Fig. 1. Chemical structure of CF.

the final concentration at 0.5% for each treatment. Control cells were treated with vehicle alone. MTT solution was then added to attain a final concentration of 0.5 mg/ml and further incubated for 5 h. The blue formazan formed was dissolved by adding DMSO and the absorbance was measured at 570 nm. After the preliminary screening, CF was chosen for further investigation on inhibiting growth of the colon cancer cells. Different cancer cells were incubated with 0–640 μM CF for 72 h. After the CF treatment, MTT assay was carried out as described above.

Effect of TRAIL on the growth of SW480 and SW620 cells was also determined by MTT assay. Briefly, 1×10^4 cells were seeded in 96-well plates and acclimatized for 24 h. The cells were then challenged with 0–100 ng/ml TRAIL for 24 h. MTT assay was performed and the sub-lethal dose of TRAIL was determined from the growth-inhibition plot.

Combinatory effect of exogenous TRAIL and CF on the growth of SW620 cells was also determined by MTT assay. After acclimatization, the cells were treated with 0–160 μM CF for 72 h. Twenty-four hours before the cells were harvested, the pre-determined sub-lethal dose of TRAIL (i.e. 12.5 ng/ml) was added into each well of the plate. MTT assay was then performed to determine the cell growth as outlined above. Synergistic effect was further assessed with the CalcuSyn software (Biosoft, Cambridge, UK), based on the principle of Chou and Talalay [13]. A combination index (CI) of 1 indicates an additive effect between two drugs. Synergism is reflected by a combination index of <1 whereas antagonism is reflected by a combination index of >1.

2.4. Determination of cytotoxicity

Cytotoxicity of CF was evaluated on both SW480 and CCD-18-Co cells using ToxiLight™ non-destructive cytotoxicity bioassay kit (Cambrex, Rockland, ME). The cancer cells (1×10^4) and normal cells (2×10^4) were seeded in 96-well plates and allowed to grow for 24 h for acclimatization. The cells were then incubated with 0–320 μM CF for 72 h. After the CF treatment, the culture medium was transferred to a 96-well white plate and incubated with the substrate solution (provided) for 10 min in dark. The chemiluminescence generated was measured by a luminometer. The toxicity of CF on the tested cell lines were expressed as percentage of cytotoxicity, which was calculated by the following equation:

$$\text{Cytotoxicity}\,(\%) = \frac{(\text{Treatment group} - \text{Low control group}) \times 100}{\text{High control group} - \text{Low control group}}$$

2.5. DNA-flow cytometric analysis

Effect of CF on apoptosis induction in SW480 cells was analyzed by Beckman Coulter Epics XL·MCL flow cytometer (Miami, FL). The cancer cells (5×10^5) were seeded and incubated for 24 h for acclimatization. The cells were then incubated with 0–160 μM CF for 72 h. After the CF treatment, the cells were harvested, washed twice with phosphate-buffered saline (PBS), and fixed in 70% ethanol at −20 °C overnight. The fixed cells were then incubated in dark for 30 min with 1 mg/ml ribonuclease and 10 μg/ml PI (both from Sigma). Red fluorescence from PI was measured at >625 nm. Cells displaying hypodiploid DNA content were quantified and regarded as the apoptotic population.

2.6. Immunoblotting and densitometry

For preparation of whole-cell lysate, the cells were harvested and suspended for 30 min on ice in an extraction buffer (20 mM Tris–HCl at pH 8.0, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.2% bovine serum albumin and 1% Triton X-100), supplemented with protease inhibitor cocktail (BD Biosciences, San Jose, CA). The lysate was then centrifuged for 15 min at $15\,000 \times g$. Aliquots of the supernatant were used for protein content determination by bicinchoninic acid assay (Pierce, Rockford, IL). To obtain mitochondrial and cytosolic fractions, the colon cancer cells were homogenized and fractionated by the Cytosol-Mitochondria Fractionation kit (Calbiochem) according to the manufacturer's instructions. The obtained proteins (30–80 μg) were then denatured in Laemmli buffer and resolved on 10 or 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis minigels, followed by electrophoretic transfer to a nitrocellulose membrane (Hybond ECL; Amersham, Buckinghamshire, UK). The membrane was blocked for 1 h in Tris-buffered saline with 0.1% Tween-20 containing 5% non-fat dry milk. Immunodetection was performed by overnight incubation at 4 °C with the primary antibody (1:1000–5000 dilution) diluted in the same blocking buffer. After extensive washings, the blot was probed with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution) for 1 h at room temperature, and the proteins were visualized by chemiluminescence (Cell Signaling Technology). Densitometric measurement of immunoblots was performed using LumiAnalyst 3.1 software (Roche Molecular Biochemicals).

2.7. Determination of mitochondrial membrane potential

Effect of CF on the mitochondrial membrane potential of SW480 cells was analyzed by flow cytometer, with the application of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbo- cyanine iodide (JC-1) (Invitrogen Molecular Probes™, Carlsbad, CA). The cancer cells (5×10^5) were seeded and incubated for 24 h for acclimatization. The cells were then incubated with 0–160 μM CF for 72 h. One million cells were then harvested and the dissociated cells were stained by 10 μg/ml JC-1 at 37 °C for 10 min. The stained cells were then washed once, resuspended with PBS, and analyzed by Beckman Coulter Epics XL·MCL flow cytometer. Red fluorescence from JC-1 aggregates was measured at 590 nm ± 17.5 nm and green fluorescence from JC-1 monomers was measured at 530 nm ± 15 nm. Data are displayed as the percentage of cells with depolarized mitochondrial membrane.

2.8. Tumor xenografts in nude mice

Male nude mice (7–8 weeks) were used in this study. All the mice were supplied by the Laboratory Animal Service Centre (LASEC) of the Chinese University of Hong Kong. The animals were housed in LASEC under the normal laboratory conditions (21 ± 2 °C, 12/12 h light/dark cycle). They were freely accessible to sterile water and maintained on standard rodent chow. All the mice were allowed to acclimatize for a week before each experiment. SW480 cell line was maintained in vitro, and was passaged twice a week. The viable cells were counted by hemocytometer and Trypan blue exclusion method. Five million cells in 0.2 ml fresh culture medium was inoculated subcutaneously onto the back of each anesthetized mouse (∼17–22 g). The tumor-bearing mice were then separated and caged randomly into different groups. Ten days after inoculation, mice in the treatment groups were administered with 1.6 or 32 mg/kg CF in 0.1 ml olive oil by gavage for 10 consecutive days. Mice in the control group received 0.1 ml olive oil only. Twenty-one days after the inoculation, the mice were first anesthetized and were then sacrificed by cervical dislocation. Tumors and liver were enucleated and weighed. All the mice were monitored and their body weight was recorded throughout the experimental period as an assessment of drug toxicity. All animal handling and experimental procedures were performed according to the regulations of the Animals (Control of Experiments) Ordinance (Department of Health, Hong Kong).

2.9. Statistical analysis

Results are expressed as mean ± standard deviation (S.D.). Difference in means between the control and treatment groups was compared by either Student's *t*-test, $p < 0.05$, or one-way ANOVA followed by Tukey's test for multiple comparisons, $p < 0.05$.

3. Results

3.1. CF inhibits growth of SW480 cells in vitro and causes regression of SW480 xenograft in vivo

Various rice bran phenolic compounds (200 μM) were incubated with a panel of human CRC cells, including SW480, SW620 and Colo-201, for 72 h. Among the phenolic compounds tested, CF showed the most prominent growth inhibition on the CRC cells. The cancer cell growth was inhibited by 62, 31 and 21% of their control levels, respectively (Fig. 2A). Therefore, CF was chosen for further investigation in the following study. The dose effect of CF (0–640 μM) on inhibiting growth of the CRC cells was then investigated. Among the CRC cells, SW480 was found to be the most responsive cell line to the CF treatment with dose-dependent growth inhibition (Fig. 2B). The anticancer effect of CF was further confirmed with nude mice transplanted with SW480 solid tumor. Administrations with CF (1.6 and 32 mg/kg) for 10 consecutive days reduced the tumor weight by 43 and 47% of the control level, respectively (Fig. 2C). During the period of treatment, neither weight loss, visible signs of toxicity, nor obvious tissue damages in vital organs, such as liver, could be detected (data not shown). The cytotoxicity of CF on SW480 and normal colon CCD-18-Co cells was then quantified by measuring activity of adenylate kinase, which was discharged from the cells into the culture medium due to the loss of cell integrity through damage to the plasma membrane. CF was more toxic to the cancer cells than to the normal cells so that the cytotoxicity was found to be 22.0 and 3.9% respectively at the 320 μM treatment (Fig. 2D). In compatible, microscopic observations demonstrated that CF reduced density of SW480 cells dose-dependently but did not alter that of CCD-18-Co cells significantly (Fig. 2E).

3.2. CF elevates expression of death receptors and induces caspase-dependent apoptosis in SW480 cells

To investigate the mechanisms of growth inhibition, SW480 cells were incubated with different doses of CF for 72 h. The cells in apoptosis were then identified with DNA-PI flow cytometry. The

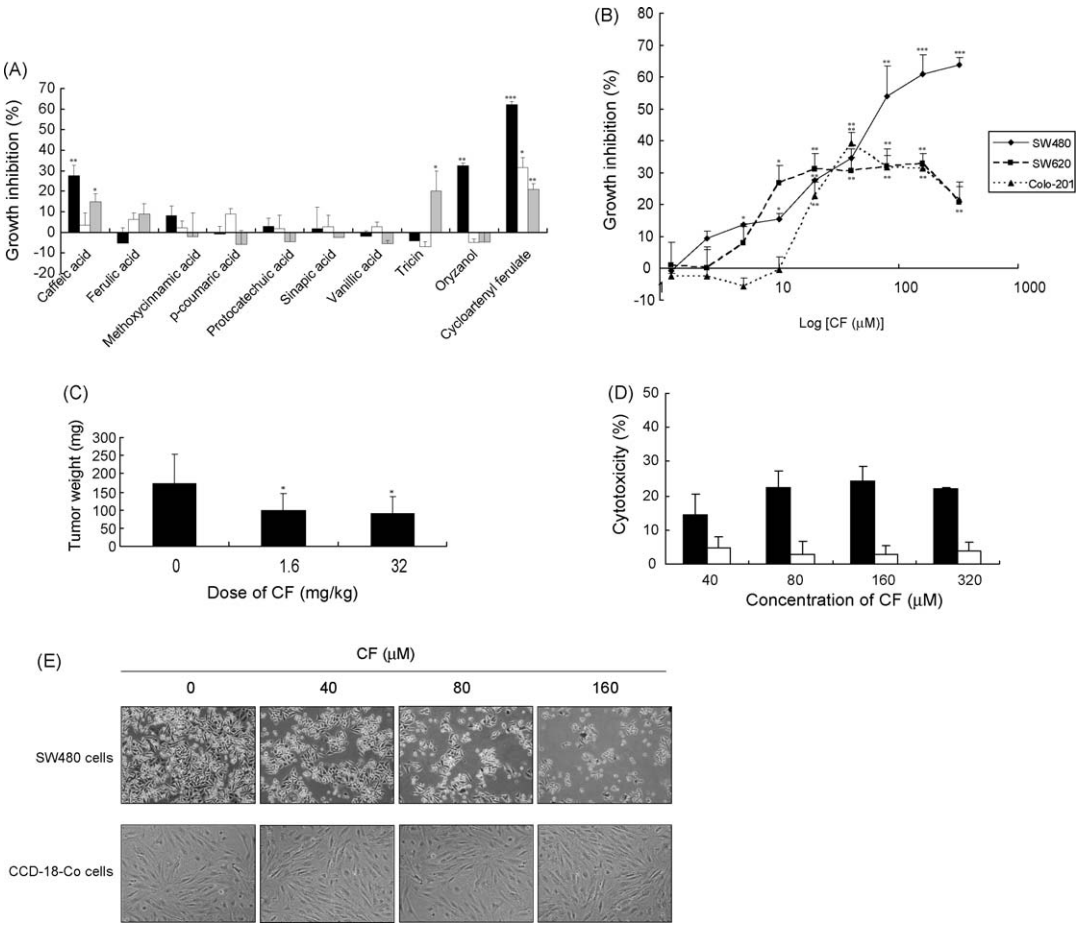


Fig. 2. CF inhibits growth of SW480 cells in vitro and induces xenograft regression in nude mice in vivo. (A) A panel of CRC cells, including SW480 (black bars), SW620 (white bars) and Colo-201 (grey bars) with increasing metastatic potential, was treated with various rice bran phenolic compounds at dosage of 200 μM for 72 h, and was then analyzed by MTT assay. (B) Dose effect of CF was investigated on the cancer cells after 72 h of incubation. Results are expressed as percentage of growth inhibition compared with their corresponding controls. Treatment caused significant increase in the percentage of growth inhibition compared with the corresponding control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (C) Ten days after inoculation of SW480 cells, the tumor-bearing mice were administered with 0, 1.6 and 32 mg/kg CF by gavage in 0.1 ml olive oil for 10 consecutive days. The weight of tumors enucleated from sacrificed mice is shown. Results are shown as mean values ± S.D.s ($n = 5$). Treatment caused significant decrease in tumor weight compared with the control ($^{*}p < 0.05$). The experiment was repeated at least twice with similar finding. (D) Cytotoxicity on SW480 (black bars) and CCD-18-Co (white bars) cells following increasing concentrations of CF treatments for 72 h was quantified by ToxiLight™ assay. Results are expressed as the percentage of cytotoxicity compared with their corresponding controls and are shown as mean values from three independent experiments ± S.D.s. (E) SW480 and CCD-18-Co cells were treated with 0, 40, 80 and 160 μM CF for 72 h and observed under inverted microscope.

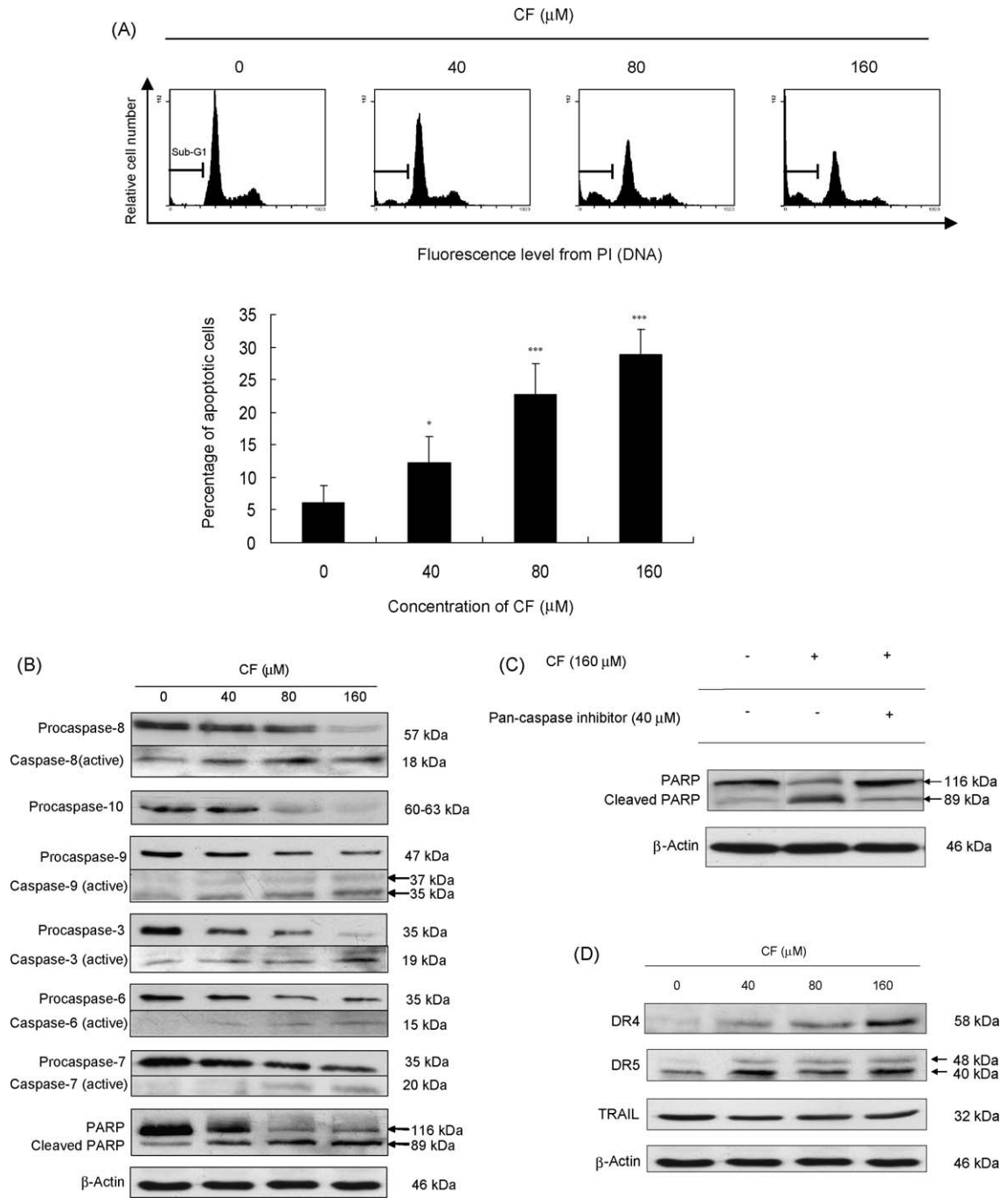


Fig. 3. CF induces caspase-dependent apoptosis and elevates expression of death receptors in SW480 cells. (A) Cells were treated with 0, 40, 80 or 160 μM CF for 72 h and subjected to flow cytometric analysis of DNA content using propidium iodide staining. Cells with hypodiploid DNA content (sub-G1 population) were counted and their numbers are expressed as a percentage of total population. Results are shown as mean values from three independent experiments ± S.D.s. Treatment caused significant increase in the percentage of apoptotic cells compared with the control ($^{*}p < 0.05$, $^{***}p < 0.001$). (B) Processing of caspases and PARP after treatment with different doses of CF was determined by western blotting. β-Actin was used as loading control. (C) Cells preincubated for 1 h in the absence or presence of 40 μM pan-caspase inhibitor, z-VAD-fmk, were treated with 160 μM CF for 72 h and analyzed by western blotting for processing of PARP. (D) The expression of death receptors (DR4 and DR5) and TRAIL with CF treatment for 72 h was analyzed by western blotting. Representative immunoblots from at least two independent experiments are shown.

apoptotic cells, which had fragmented DNA and were shown as sub-G1 in DNA histogram, were significantly increased at the dosage as low as 40 μM CF (Fig. 3A). These apoptotic cells increased from 12.2% at 40 μM to 28.9% of the total population at 160 μM CF. To further confirm the ability of CF to induce apoptosis in SW480 cells, detection of PARP cleavage, which is one of the hallmark features of apoptosis, was performed by immunoblotting. Being compatible to the finding from DNA-PI flow cytometry, 40 μM CF was first found to induce proteolytic inactivation of PARP and the extent of cleavage elevated dose-dependently (Fig. 3B). We also demonstrated that the pan-caspase inhibitor was able to rescue the cells completely from having PARP cleavage (Fig. 3C), indicating that CF elicited solely caspase-dependent apoptosis. Since PARP is a substrate of executioner caspase, its cleavage indicated that caspases might be activated during the cell death process. Caspase activation involves cleavage of its procaspase. After the CF treatment, caspase-8 and -10 of the extrinsic pathway and caspase-9 of the intrinsic pathway were activated, which were evidenced by the depletion of procaspases and the presence of the corresponding active subunits. Besides, the effector caspase-3, -6

and -7 were also activated with the CF treatment (Fig. 3B). To further investigate participation of the extrinsic pathway of apoptosis in the CF-induced cell death, expression of death receptors (DR4 and DR5) and TRAIL was evaluated. As shown in the immunoblots, expression of both DR4 and DR5 was elevated with the CF treatment while that of TRAIL remained relatively unchanged (Fig. 3D).

3.3. CF alters mitochondrial membrane potential and triggers release of apoptogenic factors

Mitochondrial membrane depolarization, which occurs in the earlier stage of apoptosis, can be detected when the mitochondria permeability transition pores open, resulting in the free distribution of ions between both sides of the membrane [14]. To elucidate whether CF had triggered the intrinsic pathway of apoptosis, mitochondrial membrane potential was measured using JC-1 and flow cytometer (Fig. 4A). Under normal circumstances, JC-1 accumulates in active mitochondria in which it oligomerizes and fluoresces red. A reduction in mitochondrial membrane potential results in the replacement of red JC-1 aggregates by green JC-1 monomers and a subsequent increase in the mean green fluorescent intensity. CF elevated the percentage of cells with depolarized mitochondrial membrane dose-dependently. The depolarized cells increased from 31.8% at 40 μM to 40.8% of the total population at 160 μM CF. It is recognized that upon firing of

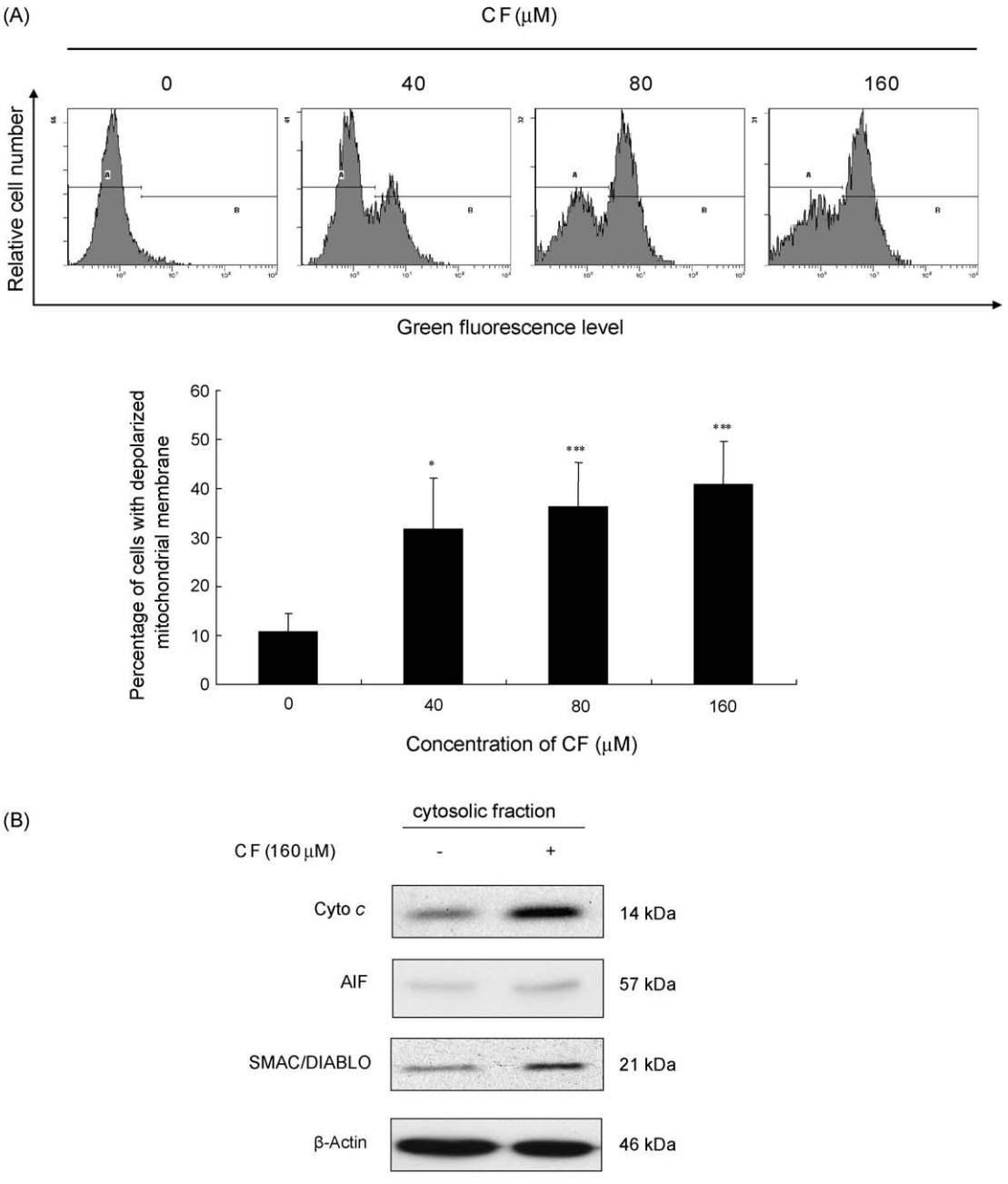


Fig. 4. CF abrogates mitochondrial membrane potential and induces release of apoptogenic factors in SW480 cells. (A) Cells treated with 0, 40, 80 or 160 μM CF for 72 h were harvested and subjected to flow cytometric analysis of mitochondrial membrane potential by JC-1 staining. Percentages of cells with depolarized mitochondrial membrane are shown. Results are shown as mean values from three independent experiments ± S.D.s. Treatment caused significant increase in the percentage of cells with depolarized mitochondrial membrane compared with the control (*$p < 0.05$, ***$p < 0.001$). (B) Homogenate from cells treated with 160 μM CF for 72 h was separated into cytosolic and mitochondrial fractions. Release of cyto *c*, AIF and SMAC/DIABLO from mitochondria into the cytosol was evaluated by western blotting of the cytosolic fraction. β-Actin was used as loading control. Representative immunoblots from two independent experiments are shown.

the mitochondrial apoptotic signaling pathway, mitochondrial membrane is first depolarized by the Bcl-2 family proteins so that apoptogenic factors, such as cytochrome *c* (cyto *c*), second mitochondrial derived activator of apoptosis (SMAC)/direct IAP binding protein with low PI (DIABLO) and apoptosis inducing factor (AIF), are released out of mitochondria. Cyto *c* binds to procaspase-9/Apaf 1 complex in the cytosol for caspase-9 activation [15], while SMAC/DIABLO interacts with the inhibitors of apoptosis protein (IAPs) to relieve the inhibitory effect on caspases [16]. AIF, on the other hand, triggers caspase-independent apoptosis by binding and causing destruction of DNA. Results from the immunoblot studies illustrated that, in conjunction with the mitochondrial membrane depolarization, cyto *c* and SMAC/DIABLO, but not AIF, were released from mitochondria into the cytosol upon the CF treatment (Fig. 4B). Taken together, these data suggest that CF causes mitochondrial dysfunction and induces caspase-dependent apoptosis in SW480 cells.

3.4. CF modulates expression of the Bcl-2 family members

Bcl-2 family proteins interact with each other and maintain a balance to regulate the mitochondrial membrane permeability. To determine if CF modulates expression of the Bcl-2 family proteins to induce the depolarization of mitochondrial membrane, expression of a number of pro- and anti-apoptotic Bcl-2 family proteins was investigated using Western blot analysis. CF depleted the anti-apoptotic Bcl-2 protein dose-dependently and elevated the pro-apoptotic Bak protein (Fig. 5A). Participation of mitochondria in the CF-induced apoptosis was further demonstrated by the cleavage and thus activation of Bid protein. As shown in Fig. 5A, CF depleted bid dose-dependently. Caspase-8 inhibitor reverted the CF-induced Bid depletion to the control level (Fig. 5B), suggesting that the decrease of Bid protein was due to cleavage and activation by caspase-8 into its truncated form. This truncated Bid (tBid), together with Bcl-2 depletion and Bak elevation, might favor the mitochondrial membrane depolarization and discharge of apoptogenic factors, including cyto *c* and SMAC/DIABLO, from mitochondria into the cytosol.

3.5. CF exhibits synergism with TRAIL in inhibiting growth of metastatic and resistant SW620 cells

We investigated the dose effect of TRAIL (0–100 ng/ml) on inhibiting growth of SW480 and SW620 cells. Results from MTT study illustrated that the growth of SW480 and SW620 was inhibited by 94.4 and 39.0% of their control levels respectively at 100 ng/ml TRAIL (Fig. 6A), indicating that SW620 was more resistant than SW480 to the TRAIL treatment. To investigate any synergism between CF and TRAIL on inhibiting growth of colon cancer cells, 12.5 ng/ml TRAIL was chosen since it alone did not inhibit the growth of SW620 cells significantly. The cells were first treated with different doses of CF for 48 h, followed by 24 h incubation with 12.5 ng/ml TRAIL. As shown in Fig. 6B, TRAIL alone did not inhibit the cell growth significantly. At 160 μM CF, the cell growth was only inhibited by about 30% of the control level. However, TRAIL together with 160 μM CF inhibited the cell growth by >50%, which was significantly higher than the inhibition induced by CF alone. The synergistic effect between CF and TRAIL was further determined quantitatively by calculating the combination index values, according to the method of Chou and Talalay [13]. We confirmed that synergism existed between CF and TRAIL, which occurred at 20, 40, 80 and 160 μM CF with CI values of 0.14, 0.113, 0.089 and 0.094, respectively (Fig. 6C). Therefore, CF exhibited synergism with TRAIL to inhibit the growth of resistant SW620 cells. To further delineate the mechanism for the synergism between CF and TRAIL, caspase-8 and -3 were immunoblotted. CF at 40 μM did not cause proteolytic cleavage and activation of procaspase-8 and -3 and TRAIL only induced their mild activation. Strikingly, more procaspase-8 and -3 were cleaved into their active forms in the SW620 cells that had been treated with both CF and TRAIL (Fig. 6D), suggesting that the synergism on growth inhibition might be due to enhanced activation of these proteolytic enzymes.

4. Discussion

Whole grain consumption has been shown to provide protection against CRC in human intervention [17]. Verschoyle et al. [18] also reported that diet with 30% rice bran significantly reduced the

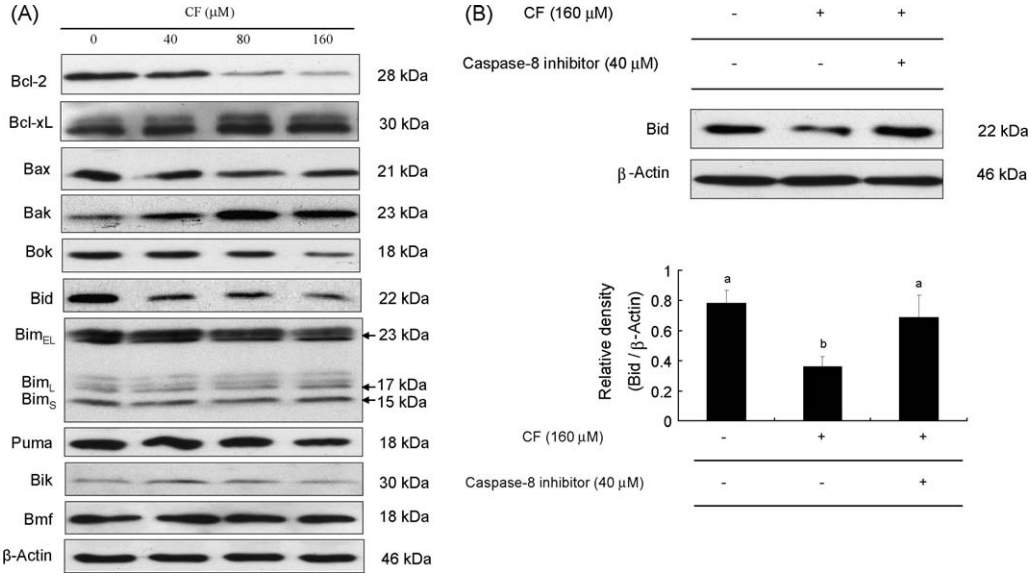
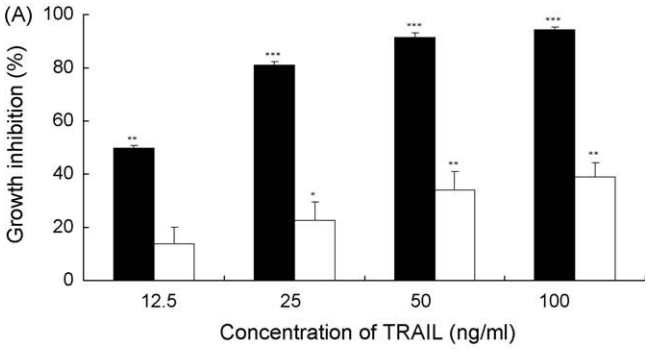
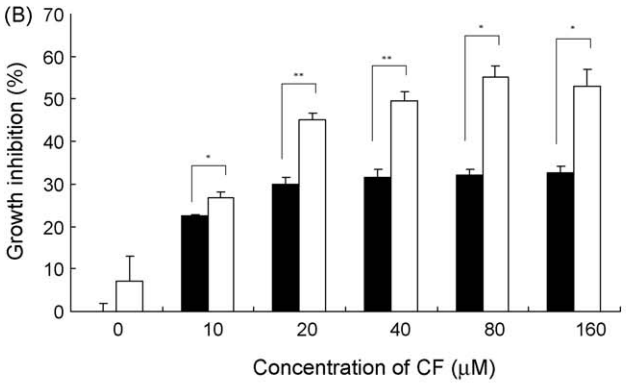


Fig. 5. CF triggers depletion of anti-apoptotic bcl-2, up-regulation of pro-apoptotic bak and processing of bid in SW480 cells. (A) Lysate from cells treated with 0, 40, 80 or 160 μM CF for 72 h was harvested and subjected to western blotting of the Bcl-2 family members. (B) Cells pre-incubated for 1 h in the absence or presence of 40 μM caspase-8 inhibitor, z-IETD-fmk, were treated with 160 μM CF for 72 h and analyzed by western blotting for processing of bid. Densitometric data indicate relative band intensity of bid with respect to that of β-actin. Results are shown as mean values from three independent experiments ± S.D.s. Bars marked by the same letter have no significant difference (Tukey's test, $p < 0.05$). β-Actin was used as loading control. Representative immunoblots from three independent experiments are shown.





(C)

TRAIL (12.5 ng/ml) + CF (μM)	CI values
10	1.179
20	0.140
40	0.113
80	0.089
160	0.094

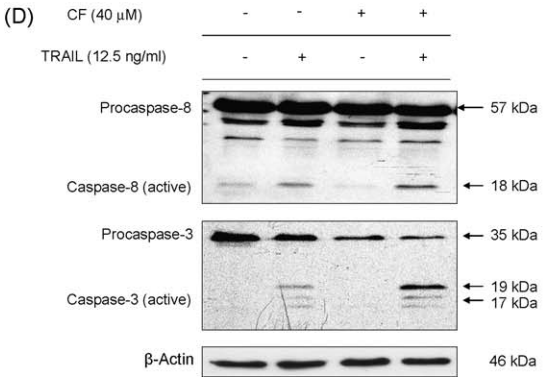


Fig. 6. CF sensitizes SW620 cells to TRAIL treatment upon enhanced activation of caspase-8 and -3. (A) SW480 (black bars) and SW620 (white bars) were treated with increasing concentrations of TRAIL for 24 h and analyzed by MTT assay. Results are expressed as the percentage of growth inhibition compared with their corresponding controls. Treatment caused significant increase in the percentage of growth inhibition compared with the corresponding control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (B) SW620 cells were pre-treated with increasing concentrations of CF for 48 h, followed by 24 h incubation without (black bars) or with the sub-lethal dose of TRAIL (i.e. 12.5 ng/ml; white bars) and analyzed by MTT assay. Results are expressed as the percentage of growth inhibition compared with their corresponding controls. Treatment caused significant increase in the percentage of growth inhibition compared with the control (i.e. treatment under the same concentration of CF; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Results are shown as mean values from three independent experiments ± S.D.s. (C) Synergy was determined for different drug combinations by calculating combination index values with CalcuSyn (as outlined in Section 2). CI > 1 denotes antagonism, CI = 1 denotes additivity, and CI < 1 denotes synergism. (D) Processing of caspase-8 and -3 with CF and TRAIL treatments in SW620 cells for 72 h was determined by western blotting. β-Actin was used as loading control. Representative immunoblots from two independent experiments are shown.

number of adenomas in Apc^{Min} mice. It is suggested that the chemopreventive properties of whole grain consumption are not attributed only to the fibers but also to other phytochemicals [6]. Several phenolic compounds including caffeic acid, cycloartenyl ferulate, ferulic acid, methoxycinnamic acid, *p*-coumaric acid, protocatechuic acid, sinapic acid, tricin and vanillic acid have been identified in the ethyl acetate extract of rice bran. Some of them have been shown to inhibit growth of human breast and colon cancer cells [19]. Rice bran oil is a rich source of bioactive polyphenols with antioxidative and cancer chemopreventive properties. It is a plant oil produced in Japan from domestic raw materials [20]. γ-Oryzanol, which consists of a mixture of esters of ferulic acid with plant sterol or triterpene alcohol, is unique to rice oil and is used commercially in Japan as a dietary supplement. In this study, the growth-inhibitory effect of various rice bran phenolic compounds was first determined on a panel of CRC cells with different metastatic potentials. We showed for the first time that CF, which is a major component present in γ-oryzanol, induced apoptosis in non-metastatic SW480 while sparing the normal colon CCD-18-Co cells. SW480, which has mutated p53 alleles, is incapable of performing apoptosis efficiently after treatment with adriamycin or etoposide [21]. Loss of functional p53 leading to apoptosis impairment and drug resistance development has been regarded as a common phenomenon in solid tumors [22,23]. Accumulating evidence demonstrates that dietary compounds offer great potential in the fight against cancer by inhibiting the carcinogenesis process through induction of apoptosis. CF was found to induce apoptosis in SW480 in vitro, evidenced by increases in DNA fragmentation and PARP cleavage. The anticancer activity of CF was further illustrated by its ability to induce regression of SW480 xenograft in nude mice. No weight loss, other visible signs of toxicity, or obvious tissue damages in the liver could be detected in the animals even at the highest dosage of treatment. All these results suggest that CF possesses anti-tumorigenic properties independent of the p53 status in the colon cancer cells. The CF-induced apoptosis is mediated by a cascade of caspases, including initiator caspase-8 and -10 of the extrinsic pathway and caspase-9 of the intrinsic pathway of apoptosis. These initiator caspases then activate effector capsase-3, -6 and -7 in SW480. Cell survival depends on the maintenance of mitochondrial membrane potential because of its involvement in ATP synthesis and maintenance of oxidative phosphorylation [24,25]. CF induces mitochondrial membrane depolarization and release of cyto *c* and SMAC/DIABLO, but not AIF, from mitochondria into the cytosol. The released cyto *c* forms apoptosome with apaf-1 so that caspase-9 is activated [26]; in addition, SMAC/DIABLO in the cytosol relieves inhibition on the activated caspases [16].

Overexpression of Bcl-2, Bcl-xL, or other anti-apoptotic homologues or loss of pro-apoptotic Bax or Bak is a common feature of human tumors [27,28]. CF elevates pro-apoptotic Bak and depletes anti-apoptotic Bcl-2 in SW480. Bcl-2 is known to inhibit release of cyto *c* and SMAC/DIABLO in human tumor cell lines [29,30]. CF-induced Bcl-2 depletion may be necessary to facilitate release of cyto *c* and SMAC/DIABLO from mitochondria. CF also depletes Bid protein level. During the apoptosis initiated by ligation of the death receptors, bid is proteolytically cleaved by caspase-8 to a pro-apoptotic form [31]. This truncated bid then interacts and activates bax or bak, which eventually causes mitochondrial membrane depolarization [32]. In order to confirm the role of Bid in the linkage between the extrinsic and the intrinsic pathways of apoptosis, SW480 cells were pre-treated with caspase-8 inhibitor prior to the CF treatment. Strikingly, caspase-8 inhibition significantly reverted the CF-induced depletion of Bid to the control level, suggesting that processing and thus activation of bid may play a crucial role in the mitochondrial amplification step for full activation of caspase cascade and apoptosis induction. The

extrinsic pathway, which originates at the membrane and engages cell surface death receptors, is shown to depend upon upstream caspase-8 activation [33,34]. Activation of caspase-8 in the CF-treated SW480 may occur upstream at the level of death receptors. TRAIL, which belongs to TNF superfamily of cytokines, is currently in phase I clinical trials [12] whereas the other family members, including TNF-alpha and FasL, cannot enter the clinic due to their toxic nature [34]. CF elevates both DR4 and DR5, but not TRAIL, in SW480. Delmas et al. [35] reported that resveratrol triggered apoptosis in SW480 by inducing death receptor Fas aggregation and Fas activation independent of FasL. Nevertheless, the CF-induced DR4/DR5 aggregation needs to be demonstrated and its role in the apoptotic process remains to be determined. Although there is no TRAIL elevation, we speculate that CF-induced DR4 and DR5 up-regulation may be sufficient to cause death receptor aggregation and the death-inducing signaling complex (DISC) formation so that both caspase-8 and -10 can be activated.

Besides acting as a chemopreventive compound to halt the progression of cancer cells to a later stage of carcinogenesis, CF can also sensitize metastatic SW620 cells to TRAIL-induced apoptosis. Previous studies have shown that DR4 and DR5 levels correlate with TRAIL sensitivity in some tumor cell lines [36–38]. Since CF elevates both DR4 and DR5 in SW480, we are interested to know whether CF could sensitize SW620, which is the metastatic form of SW480 derived from the same patient at a later stage of carcinogenesis, to TRAIL-induced growth inhibition. TRAIL is a promising therapeutic agent that induces apoptosis selectively in cancer cells; however, intrinsic or acquired resistance has been observed in several colon cancer cells that results in limited efficiency of TRAIL treatment [39,40]. Dietary carotenoids have been shown to up-regulate DR5 expression and to sensitize the colorectal cancer DLD-1 cells to TRAIL-induced apoptosis [41]. In addition, quercetin was found to induce a clustered distribution of death receptors into raft domains of plasma membrane and to enhance TRAIL-mediated apoptosis in human colon adenocarcinoma HT-29 cells [42]. We show in this study that SW620 is more resistant than SW480 to TRAIL treatment. Interestingly, CF cooperates with TRAIL to inhibit growth of the metastatic SW620 through at least enhanced activation of caspase-8 and -3. It is not yet clear whether CF is capable of redistributing the death receptors to sensitize SW620 to TRAIL-induced apoptosis. Further studies are deserved to acquire a better understanding of the molecular mechanisms responsible for the sensitization effect.

In summary, we demonstrate that CF is capable of triggering apoptosis in early stage CRC cells. Furthermore, the synergism between CF and TRAIL may open a new research direction that dietary chemopreventive compound may act as an adjuvant to sensitize metastatic CRC cells, which some of them have developed a number of resistance mechanisms and compromised the efficacy of TRAIL treatment.

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