Mechanisms of Grape Seed Procyanidin-induced Apoptosis in Colorectal Carcinoma Cells

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Abstract. *Background: Grape seed procyanidins (GSP) can inhibit cell proliferation and tumorigenesis, and induce apoptosis in human breast, prostate, skin and colorectal carcinoma cell lines. Materials and Methods: In order to study the mechanism of apoptosis, four colorectal cell lines, HT-29, SW-480, LoVo and Colo 320DM, were used. GSP-treated cells were assessed for viability by trypan blue exclusion, for loss of mitochondrial membrane potential by rhodamine 123 staining, for increased apoptosis by annexin V labeling, and for changes in the levels of proteins involved in apoptosis by immunoblotting. Results: GSP had no significant pro-apoptotic effect on the Colo 320DM cell line. In HT-29, SW-480 and LoVo cells, GSP (12.5-50 mg/l) inhibited proliferation in a dose-dependent manner. In these three lines, GSP treatment increased the proportion of rhodamine 123-negative cells and annexin V-positive cells, while immunoblotting revealed increased levels of apoptosis activation protein, caspase-3 and the cleavage fragment of PARP (a caspase-3 substrate), but the level of Bcl-2 did not change. Conclusion: GSP inhibited the proliferation of some colorectal carcinoma cell lines and was associated with an apoptotic mechanism involving a loss of mitochondrial membrane potential and caspase-3 activation in these cells.*

During the period that the Taiwanese diet became more Westernized, colorectal carcinoma (CRC) became the third most common cancer diagnosed both in men and women in Taiwan. Many epidemiological studies and animal experiments have suggested that vegetables, fruits, and their constituents protect against cancer (1-4) and that this protection may partly be attributed to polyphenolic compounds, which have antioxidant and free radical-scavenging properties (5-7). Grape seed procyanidin (GSP) is a nutritional supplement with known disease prevention characteristics (8-11). GSP has both chemopreventative and antiproliferative effects on breast, prostate, skin and colorectal cancer cells (12-19), indicating a potential for application in cancer management.

CRC is a diverse disease with a remarkable ability to develop resistance to chemotherapy. One of the main mechanisms of chemotherapy resistance in CRC cells is the development of insensitivity to apoptosis induction (20). Previous studies demonstrated that GSP exhibited strong apoptotic induction in some CRC cell lines, including HT-29, LoVo and CaCo2 (17, 18). The apoptotic activity induced by GSP was recognized to be mainly mediated by the activation of caspase-3. Recently, the activation of caspase-3 by GSP has been confirmed by the loss of the integrity of the mitochondrial membrane in breast and skin cancer cells (19, 21). After the destruction of the mitochondrial membrane, the mitochondrial protein cytochrome *c* is released into the cytoplasm, which participates in the apoptosome and the cleavage of procaspase-3 into its active form. Although apoptotic activation in CRC by GSP has been reported (17, 18), it is still unknown whether the actual mechanism of apoptosis is mediated by loss of the integrity of the mitochondrial membrane or not. The current study investigated the effect of GSP in 4 different CRC cell lines in order to elucidate the possible mechanism of apoptotic induction in CRC after GSP treatment.

Materials and Methods

Materials. Media (RPMI, McCoy's 5a, MEM and L-15 media) fetal bovine serum, L-glutamine, trypsin and antibiotics were purchased from Gibco Ltd (Paisley, UK). Proteinase inhibitor cocktail, sodium orthovanadate, NaF, sodium pyrophosphate, Triton® X-100, ammonia persulfate, *N,N,N',N'*-tetramethylethylenediamine (TEMED), Tween

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20 and rhodamine 123 were from Sigma (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL, USA). Acrylamide was from Bio-Rad (Hercules, CA, USA). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was from Millipore (Bedford, MA, USA). Mouse monoclonal anti-caspase-3 and -Bcl-2 antibodies were from Zymed (San Francisco, CA, USA). Goat anti-PARP antibody and goat anti-rabbit and anti-mouse secondary antibodies conjugated with peroxidase were from R&D Systems (Minneapolis, MN, USA). X-ray film was from Fuji (Tokyo, Japan). The GSP powder was from Puritan's Pride Inc. (Oakdale, NY, USA). Annexin V conjugated with FITC was from Gene Research (Taipei, Taiwan).

Cell lines. Human CRC cell lines SW480, Colo 320DM, LoVo and HT-29 were obtained from the Bioresource Collection and Research Center in Taiwan. The culture conditions were those recommended by the Bioresource Collection and Research Center. All experiments were carried out on cell lines passaged between 5-20 times.

Determination of phytochemicals in GSP. The amount of total phenolics in GSP was estimated by the Folin-Ciocalteu method essentially as described elsewhere (22) Briefly, 0.25 ml diluted sample was added to 3.5 ml of distilled water in screw-capped test tubes followed by the addition of 0.5 ml of Folin-Ciocalteu solution. After 3 min, 1 ml of sodium carbonate (20%) was added, and the test tubes were shaken well before they were incubated in a boiling water-bath for 1 min. The tubes were then allowed to cool in darkness. A blue coloration developed and the absorbance was read at 685 nm. Results were expressed in mg of gallic acid equivalent/g dry mass GSP.

The total proanthocyanidin was determined by acid/butanol assay. A 0.25 ml aliquot of GSP extract was added to 3 ml of a 95% solution of *n*-butanol/HCl (95:5 v/v) in stoppered test tubes, followed by the addition of 0.1 ml of a solution of NH_4Fe $(SO_4)_2 \cdot 12H_2O$ in 2 M HCl. The tubes were incubated for 40 min at 95°C. A red coloration was developed and the absorbance was read at 550 nm. The proanthocyanidin content was expressed in mg of cyanidin chloride equivalent/g dry mass of GSP.

The total flavonoid content was measured by the $AlCl_3$ method. Aliquots of 1.5 ml of GSP were added to equal volumes of a solution of 2% $AlCl_3 \cdot 6H_2O$ (2 g in 100 ml methanol). The mixture was vigorously shaken and absorbance was read at 367.5 nm after 10 min of incubation. Flavonoid content was expressed in mg catechin equivalent/g dry mass of GSP.

Condensed tannins were determined by the vanillin method essentially as described elsewhere (17). An aliquot of GSP was added to a final volume of 5 ml of vanillin reagent (0.5% vanillin in glacial acetic acid containing 4% HCl). Catechin was used as a standard. The absorbance of the pink chromogen formed was read at 510 nm. The concentration of condensed tannins was expressed as mg catechin equivalent/g dry mass of GSP.

Cell proliferation assay. SW480, HT-29, LoVo and Colo 320DM cells were plated at 100,000 cells in 60-mm tissue culture dishes. After 18 h of culture, cells were treated with GSP (0, 12.5, 25, or 50 mg/l). At 24, 48, and 72 h, cells were collected by trypsinization, stained with trypan blue and suspensions were counted in duplicate using a hemocytometer. Data were taken from averages of three independent experiments.

Mitochondrial membrane potential ($\Delta\phi_m$). $\Delta\phi_m$ measurement was carried out essentially as described elsewhere (23). Briefly, the GSP-treated cells were harvested and suspended at a density of 1×10^6 cells per ml in fresh medium. The cells were then stained with rhodamine 123 at a final concentration of 10 mg/l for 30 min at 37°C. Cells were then washed twice with fresh medium and the fluorescence intensity of cells was immediately examined by flow cytometry (Becton Dickinson, CA, USA). Ten thousand cells without cell debris were analyzed and the rhodamine 123-negative cells were defined as the cells whose fluorescence intensity was lower than that of untreated cells.

Apoptosis. Measurement of apoptosis was carried out by using annexin V to label the cell surface phosphatidylserine, which is a biomarker of apoptotic cells. Briefly, the treated cells were trypsinized and washed twice with phosphate-buffered saline, then suspended in binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl and 2.5 mM $CaCl_2$). Cells were stained with 5 μl annexin conjugated with fluorescein isothiocyanate (FITC) at room temperature in the dark for 30 min. The cells were analyzed by flow cytometry to measure the fluorescence intensity using the FL-1H channel to detect FITC. Untreated cells served as the negative control.

Immunoblotting. CRC cells were washed twice with ice-cold phosphate-buffered saline and lysed in 0.5 ml of homogenization buffer (10 mM Tris-HCl at pH 7.4, 2 mM EDTA, 1 mM EGTA, 50 mM NaCl, 1% Triton® X-100, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 1:100 *v.v* proteinase inhibitor cocktail) at 4°C for 30 min. Cell lysates were then ultracentrifuged at 100,000 g for 30 min at 4°C and the supernatants were used as the cell extracts. Protein concentration of the cell extract was determined by BCA protein assay and the extracts were adjusted to 2 mg/ml with homogenization buffer. For immunoblot analysis, the cell extract was subjected to 12% SDS-PAGE and the resolved bands were electrotransferred to PVDF membranes with a semi-dry blot apparatus (Bio-Rad) at 3 mA/cm^2 of the gel in transfer buffer (25 mM Tris (pH 8.3), 192 mM glycine and 20% methanol) at room temperature for 30 min. The free protein-binding sites on the PVDF membrane were blocked by incubation with 5% nonfat milk in TTBS buffer (20 mM Tris at pH 7.4, 0.15 M NaCl and 0.2% Tween-20) at 25°C for 2 h. The membrane was immunoblotted with 0.1 μg/ml primary antibody in TTBS buffer containing 3% nonfat milk at 4°C overnight and then with secondary antibody conjugated with peroxidase (1:1,000) at 25°C for 1 h. Immunoblots were developed using an enhanced chemiluminescence system (24). The luminescence was visualized on X-ray film.

Statistical analysis. All data are expressed as mean ± SD, unless stated otherwise. The differences between groups were calculated using the Student's unpaired *t*-test. A *p*<0.05 was regarded as statistically significant.

Results

Analysis of phytochemicals in GSP. The phytochemicals (polyphenols, flavonoids, condensed tannins and proantho-cyanidins) in GSP used here were determined by colorimetry. The content of total phenol in GSP was 658.7±4.3 mg gallic acid equivalent/g of dry mass GSP. The amounts of total
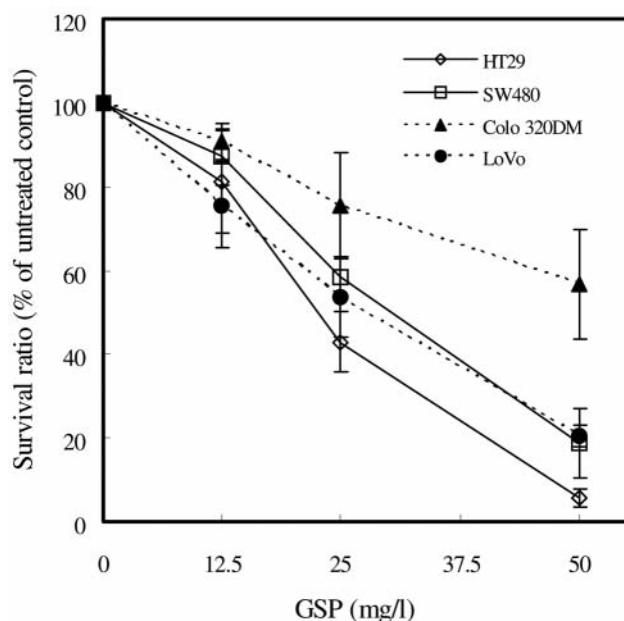
Figure 1. *The dose-dependent response to GSP in HT-29, SW480, LoVo and Colo 320DM cells. Cells were treated with increasing concentrations of GSP as indicated then incubated at 37°C for 72 h. The viable cells were stained with trypan blue and counted under a microscope. The cell viability was expressed as a percentage of untreated cells. Data are taken from the averages of three independent experiments and expressed as means ± SD.*
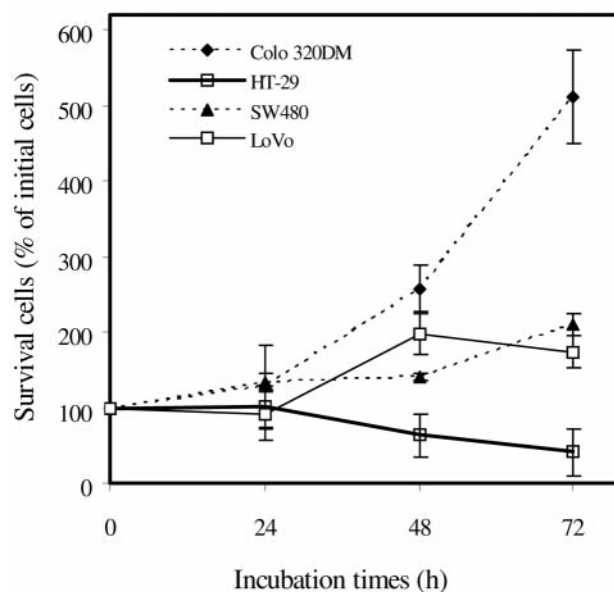

Figure 2. *The time-dependent response to GSP in HT-29, SW480, LoVo and Colo 320DM cells. The cells were treated with 12.5 mg/l GSP and trypsinized after 24, 48, and 72 h treatment. The viable cells were stained with trypan blue and counted under a microscope. The cell viability was expressed as a percentage of untreated cells. Data are taken from the averages of three independent experiments and expressed as means ± SD.*

flavonoid and of condensed tannin in GSP were 193.5±7.6 and 127.8±3.1 mg catechin equivalent/g of dry mass GSP. The content of proanthocyanidin was 31.6±5.8 mg cyanidine chloride equivalent/g of dry mass GSP. These results indicate that the GSP used here was a polyphenol-rich substance primarily containing flavonoids and condensed tannins.

The response of SW480, Colo 320DM, HT-29 and LoVo cells to GSP. The cell survival ratio was first tested. As shown in Figure 1, the cell survival ratio, compared with untreated cells, decreased in a dose-dependent manner in all four cell lines tested after incubation for 72 h. HT-29 was very sensitive to GSP: the cell survival ratio decreased to less than 10% that of the control level after 50 mg/l GSP (p<0.05). In SW480 and LoVo cells, the survival ratio also significantly (p<0.05) decreased to 20% that of the control level using 50 mg/l GSP. However, the response of Colo 320DM cells to 50 mg/l GSP was insignificant (p=0.25) and the survival ratio only decreased to 60% that of the control level.

The time-dependent response (24, 48, 72 h) of each cell line to 12.5 mg/l GSP is shown in Figure 2. The number of Colo 320DM cells increased to five times the original cell number even after treatment with a low dosage of GSP. In
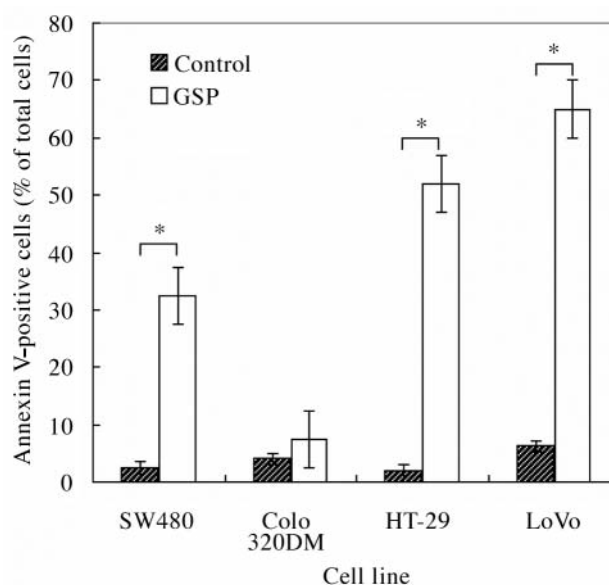

Figure 3. *Detection of phosphatidylserine translocated to cell surface. Cells were treated with 50 mg/l GSP for 72 h and then stained with annexin V conjugated with FITC. Ten thousand cells were analyzed by flow cytometry. Data are taken from the averages of three independent experiments and expressed as means ± SD. *Represents a significant difference (p<0.05).*
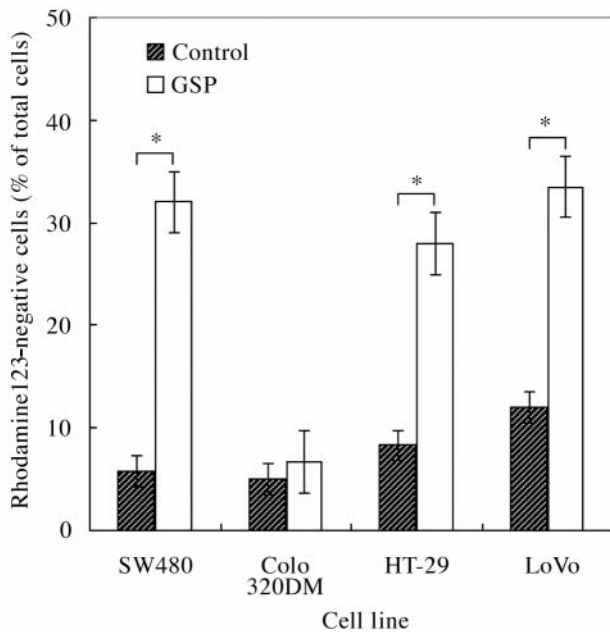
Figure 4. $\Delta\phi_m$ lost in GSP-treated cells. Cells were treated with 50 mg/l GSP for 72 h, and then stained with rhodamine 123 to mark intact mitochondria. Ten thousand cells were analyzed by flow cytometry. Data are taken from the average of three independent experiments and expressed as means ± SD. *Represents a significant difference (p<0.05).

contrast, the SW480, HT-29, and LoVo cell lines presented retarded cell proliferation in response to low dosage GSP treatment.

The apoptotic death of CRC cells induced by GSP. To determine the apoptosis-inducing effect of GSP on these CRC cells, CRC cells treated with 50 mg/l GSP were stained with annexin V-FITC and then analyzed by flow cytometry. Compared with control groups, the proportion of annexin V-positive cells of GSP-treated SW480, HT-29 and LoVo cells increased significantly ($p<0.05$) up to approximately 30%, 50% and 60% of total cells, respectively (Figure 3). Only Colo 320DM cells had few apoptotic cells, with <10% positive cells in this apoptosis assay, demonstrating that Colo 320DM cells were resistant to GSP-induced apoptosis.

The loss of $\Delta\phi_m$ in GSP-treated CRC cells. To investigate whether the apoptosis induced by GSP in CRC cells involved the loss of mitochondrial integrity, the $\Delta\phi_m$ in GSP-treated cells was analyzed. As shown in Figure 4, the percentage of rhodamine 123-negative cells of the control groups was all below 15% of total cells. After treatment with 50 mg/l GSP, the percentage increased significantly ($p<0.05$) to about 30% in SW480, HT-29 and LoVo cells. But the change in Colo 320DM cells was not remarkable and they retained their mitochondrial membrane potential.

The protein levels of Bcl-2, caspase-3 and PARP in GSP-treated CRC cells. As shown in Figure 5A, SW480 increased the caspase-3 proenzyme level after 24 h of treatment with GSP, which gradually decreased after 48 and 72 h of treatment. Active caspase-3 cleaves PARP into 23 kDa fragments; these were increased following treatment with GSP after 48 and 72 hours. The alteration of expression level of antiapoptosis protein Bcl-2 in GSP-treated SW480 cells was not remarkable. Similar results were obtained in HT-29 (Figure 5B) and LoVo (Figure 5C) cells, with the exception of the time of appearance of cleaved PARP. In contrast to these three cell lines, the caspase-3 level did not alter significantly in GSP-treated Colo 320DM cells, and no cleaved PARP could be detected (Figure 5D). Furthermore, the level of the antiapoptosis protein Bcl-2 was increased in Colo 320DM cells after GSP treatment.

Discussion

In the current study, we investigated GSP efficacy with different CRC cell lines including SW480, HT-29, LoVo and Colo 320DM, and found various responses in these cells. The proliferation of SW480, HT-29 and LoVo were systematically inhibited by GSP in a dose-dependent manner. The growth of these carcinoma cells was also retarded in a time-dependent manner by GSP after 2-3 days', culture. However, the response of Colo320DM cells to GSP was not significant. The antiproliferative results were the same as those reported by Kaur *et al.* (17) for the HT-29 and LoVo cell lines. A similar effect was also obtained in the SW480 CRC cell line, which was originally derived from a patient with a Dukes' B tumor. On the contrary, testing of the Colo 320DM cell line revealed antiproliferative resistance to the GSP treatment, indicating that there are other mechanisms existing in this carcinoma cell line that influence its response to GSP. In several reports, the GSP effect on the inhibition of proliferation of cancer cell lines was suggested to be caused by apoptosis (6, 12-19, 21). Therefore, resistance to apoptosis might represent the main mechanism by which cancer cells, such as Colo 320DM, overcome the GSP treatment. In our study, GSP treatment significantly increased the proportion of annexin V-positive cells in the HT-29, LoVo and SW480 lines, whereas the Colo 320DM line did not show significant elevation of positive cells. These results associated with apoptosis are consistent with previous reports.

It has recently been reported that the mechanism of GSP-induced apoptosis of cancer cells involves mitochondria in metastatic breast carcinoma 4T1 cells and skin carcinoma A431 cells (19, 21). Another study on the mechanism of GSP-induced apoptosis agrees with their results in HT-29, LoVo and CaCo-2 CRC cells (17, 18). However, evaluation of Colo 320DM cells did not reveal a
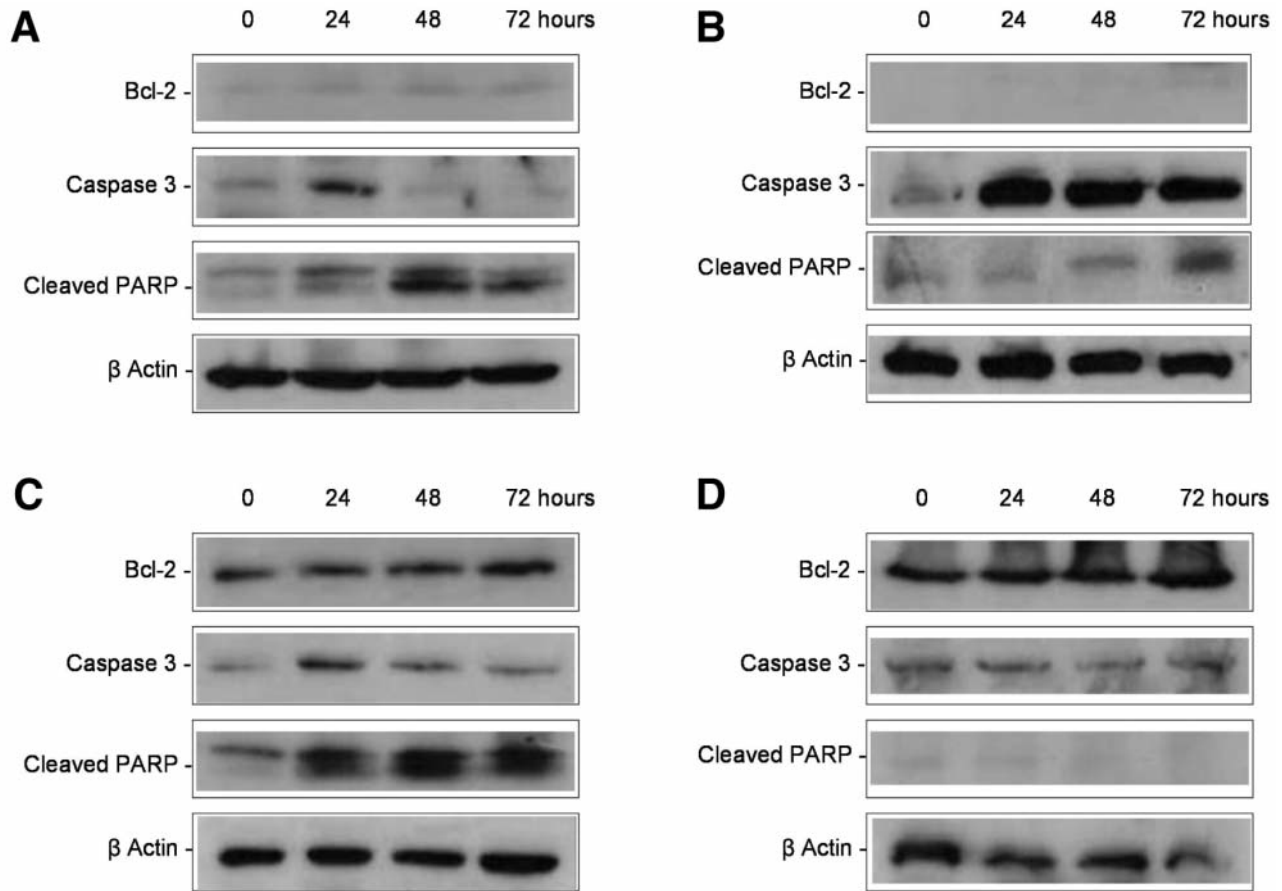
Figure 5. *Immunoblots of apoptosis-linked proteins in GSP-treated colorectal carcinoma cells. Cells were treated with 50 mg/l GSP and harvested at 24, 48, and 72 h. Cell protein lysates from SW480 (A), HT-29 (B), LoVo (C) and Colo 320DM (D) cells were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted to show levels of Bcl-2 , caspase 3, intact/cleaved PARP levels and cleavage, and beta-actin levels as loading control.*

similar effect in our study. Furthermore, we evaluated the GSP-treated cells for staining by rhodamine 123, which is sequestered by living cells with normal $\Delta\phi_m$. The untreated control groups of all four tested CRC cell lines showed strong fluorescence by flow cytometry. In contrast, the $\Delta\phi_m$ of HT-29, LoVo and SW480 cells was lost after GSP treatment, indicating that the GSP-induced apoptosis in these CRC cells was mediated by a mitochondrial pathway. Colo320DM cells did not show significant alteration of their $\Delta\phi_m$ after GSP treatment. This implied that the resistance to GSP of Colo 320DM cells came from some mechanism protecting mitochondrial integrity during GSP treatment.

The Bcl-2 family of proteins is important in mediating apoptosis in CRC cells (25, 26). Some family members promote apoptosis (*e.g.* Bax and Bad) while others inhibit it (*e.g.* Bcl-2 and Bcl-X). These proteins form multimers which act as pores in cell membranes, controlling the flux of

molecules between cellular compartments (27). Bcl-2 inhibits apoptosis by inhibiting the release of cytochrome *c* (Apaf 2) and apoptosis inducing factor (AIF) from the mitochondria to the cytoplasm and by limiting the activation of caspase-3 through inhibition of its activator protein, Apaf 1 (28). Previous reports have shown that GSP inhibits the overexpression of Bcl-2 protein in breast and skin carcinoma cells (19, 21). However, in the current study, only two untreated CRC cell lines (SW480 and Colo 320DM) showed high levels of Bcl-2. Untreated HT-29 and LoVo Cells showed little Bcl-2 protein by immunoblot. Further investigation showed that the Bcl-2 level in GSP-sensitive cell lines (SW-480, HT-29 and LoVo) did not alter after 72 h of treatment. On the contrary, the GSP-resistant cell line, Colo 320 DM, showed elevation of Bcl-2 levels during GSP treatment. The resistance to GSP-induced apoptosis might be explained in Colo 320DM cells by this progressive increase of Bcl-2.

The crucial step in apoptosis of CRC cells induced by curcumin and gypenosides is the activation of caspase-3 (29-31). Many reports also suggest that the activation of caspase-3 is the common event during GSP-induced apoptosis of CRC cells (17, 18). Our results are consistent with their reports in three cell lines. The GSP treatment increased the protein levels of uncleaved caspase-3 in SW480, HT-29, and LoVo cells after 24 h GSP treatment. The level of caspase-3 in these three cell lines gradually decreased after 48 h GSP treatment, indicating that cleavage of caspase-3 activates the enzyme pathway. The evidence for caspase-3 activation is the subsequent increase in cleavage of the caspase-3 substrate PARP in GSP-treated SW480, HT-29 and LoVo cells. However, the level of cleaved PARP did not change in the GSP-treated Colo 320DM cells, suggesting caspase-3 activation was not induced by GSP in this cell line. Elevation of the Bcl-2 level and concomitant suppression of caspase-3 activation might be the main factors behind Colo 320DM resistance to GSP-induced apoptosis.

In conclusion, GSP inhibited cell proliferation and induced apoptosis in some, but not all of the CRC cells. The mechanism of apoptosis involved loss of mitochondrial membrane potential and increased levels and activation of caspase-3. The mechanism conferring insensitivity to GSP seemed to involve an increase in Bcl-2 level and suppression of caspase-3 activation in response to GSP treatment. GSP may be a potent chemopreventive agent for some types of CRC, however, as our study presented, it was not effective on all CRC cell lines. Further investigation of diagnostic factors which can separate CRC tumors into GSP-sensitive and -insensitive groups will improve the clinical usefulness of GSP. Future studies of GSP activity should be controlled for variability in sensitivity to GSP between individual tumors and cell lines.

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