Grape Seed Extract Inhibits *In vitro* and *In vivo* Growth of Human Colorectal Carcinoma Cells

Manjinder Kaur,[1] Rana P. Singh,[1] Mallikarjuna Gu,[1] Rajesh Agarwal,[1,2] and Chapla Agarwal[1,2]

Abstract

Purpose: Accumulating evidences suggest the beneficial effects of fruit-and-vegetable consumption in lowering the risk of various cancers, including colorectal cancer. Herein, we investigated the *in vitro* and *in vivo* anticancer effects and associated mechanisms of grape seed extract (GSE), a rich source of proanthocyanidins, against colorectal cancer.

Experimental Design: Effects of GSE were examined on human colorectal cancer HT29 and LoVo cells in culture for proliferation, cell cycle progression, and apoptosis. The *in vivo* effect of oral GSE was examined on HT29 tumor xenograft growth in athymic nude mice. Xenografts were analyzed by immunohistochemistry for proliferation and apoptosis. The molecular changes associated with the biological effects of GSE were analyzed by Western blot analysis.

Results: GSE (25-100 μg/mL) causes a significant dose- and time-dependent inhibition of cell growth with concomitant increase in cell death. GSE induced G_1 phase cell cycle arrest along with a marked increase in Cip1/p21 protein level and a decrease in G_1 phase–associated cyclins and cyclin-dependent kinases. GSE-induced cell death was apoptotic and accompanied by caspase-3 activation. GSE feeding to mice at 200 mg/kg dose showed time-dependent inhibition of tumor growth without any toxicity and accounted for 44% decrease in tumor volume per mouse after 8 weeks of treatment. GSE inhibited cell proliferation but increased apoptotic cell death in tumors. GSE-treated tumors also showed enhanced Cip1/p21 protein levels and poly(ADP-ribose) polymerase cleavage.

Conclusions: GSE may be an effective chemopreventive agent against colorectal cancer, and that growth inhibitory and apoptotic effects of GSE against colorectal cancer could be mediated via an up-regulation of Cip1/p21.

Epidemiologic studies have shown that consumption of fruits and vegetables based diet reduces the risk of cancer, especially cancers of digestive tracts (1). Based on these observations, the latest global strategy on the prevention of cancer recommends "five-a-day" consumption of colorful fruits and vegetables (2). Consequently, the focus of cancer research in recent years has been shifting towards the isolation and characterization of potential chemopreventive agents present in fruits and vegetables (3). In this regard, many phytochemicals of diverse chemical nature, such as bioflavonoids, proanthocyanidins, and phytoestrogens, have shown promising chemopreventive

and/or anticancer efficacy in various cell culture and animal models (4). A rich source of proanthocyanidins is grape seed extract (GSE), which was studied for its anticancer activity against colorectal cancer in the present investigation. Most of the beneficial health effects of proanthocyanidins and GSE are attributed to their antioxidant and free radical scavenging properties (5–9). GSE is widely marketed as a dietary supplement and is considered safe for human consumption (10). In several ongoing studies by us and others, GSE has been shown to reduce the incidence of carcinogen-induced mammary tumors in rats and skin tumors in mice and to inhibit the growth of human cancer cells of varied phenotypes *in vitro* and *in vivo* (11–16).

Colorectal cancer is the second most common malignancy and cause of cancer-related deaths in the United States, and according to American Cancer Society estimates, ~145,290 new cases and 56,290 deaths would have occurred from colorectal cancer in the year 2005 (17). Progression of the disease from benign colorectal adenoma to malignant carcinoma involves accumulation of molecular alterations, including chromosomal abnormalities, genetic mutations, and epigenetic changes over a period of time (18–21). The long latency period for the development of full-blown disease provides a much needed opportunity for the intervention of colorectal cancer employing potential chemopreventive strategies. In this context, identification and/or development of chemopreventive

agents, which selectively target molecular events linked to cancer progression, could be an effective approach (22).

One of the hallmarks of cancer is uncontrolled proliferation due to loss of checkpoint control accompanying unchecked activation of cyclin-dependent kinases (CDK) responsible for cell cycle progression (23). In addition to CDKs, cyclins and CDK inhibitors (CDKI) are the key molecules that play important role in cell cycle progression (24). The levels of these cell cycle molecules are regulated quantitatively and qualitatively for normal progression through various phases of the cell cycle (25, 26). Escape of the cells from the normal cell cycle regulation results in uncontrolled cell proliferation; one of the defects found in almost every cancer, including colorectal cancer (27). Furthermore, cancer cells also acquire alterations for enhanced survival and become apoptosis resistant to anticancer therapies (28). Therefore, induction of cell cycle arrest and apoptosis by chemopreventive agents could be an effective approach to check uncontrolled cell proliferation and survival in tumor cells.

Recently, grape seed proanthocyanidins are reported to have chemopreventive efficacy against carcinogen-induced intestinal and colorectal cancers in rats (14, 29). In this study, we investigated anticancer activity and associated mechanism of GSE against human colon carcinoma cells, both in cell culture and xenograft studies. Our findings show that GSE induces cell cycle arrest and apoptosis in these cancer cells and inhibits tumor xenograft growth. Furthermore, GSE showed a marked increase in CDK inhibitor Cip1/p21 protein level, which could be a critical molecular target for GSE in both *in vitro* and *in vivo* efficacy against colorectal cancer.

Materials and Methods

Chemicals and reagents. GSE-standardized preparation, constituting of 89.3% (w/w) procyanidins, 6.6% of monomeric flavonols, 2.24% of moisture content, 1.06% of protein, and 0.8% of ash (30), was a kind gift from its commercial vendor Kikkoman Corp. (Noda City, Japan). Primary antibodies to CDK2, CDK4, CDK6, cyclin D1, cyclin E, and cyclin A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-Cip1/p21 antibody was from Upstate Biotechnology (Lake Placid, NY), and anti-Kip1/p27 was from Neomarkers, Inc. (Fremont, CA). Antibody for β-actin was from Sigma (St Louis, MO). Anti–proliferating cell nuclear antigen (anti-PCNA) antibody and streptavidin-conjugated horseradish peroxidase were from DAKO (Carpinteria, CA). Annexin V-Vybrant apoptosis kit was from Molecular Probes (Eugene, OR). Terminal deoxynucleotidyl transferase–mediated nick-end labeling kit/Tumor TACS *In situ* Apoptosis Detection kit was from R&D Systems (Minneapolis, MN). 3,3′-Diaminobenzidine and Harris hematoxylin was from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Human colorectal cancer LoVo and HT29 cell lines were purchased from the American Type Culture Collection (Manassas, VA). LoVo cells were cultured in F-12 Nutrient Mixture (HAM) with 10% fetal bovine serum (Hyclone, Logan, UT), and HT29 cells were cultured in DMEM with 10% fetal bovine serum under standard culture conditions (37°C, 95% humidified air, and 5% CO_2).

Cell growth and death assay. LoVo or HT29 cells were plated at a cell density of $5,000/cm^2$ in 60-mm culture plates under the standard culture conditions overnight. Cells were treated subsequently either with DMSO alone or with varying concentrations of GSE (0-100 μg/mL) in DMSO. At the end of desired treatment times (12-48 hours), cells were harvested by brief trypsinization and counted

using a hemocytometer. Trypan blue dye exclusion was used to differentiate between live and dead cells.

Flow cytometry analysis for cell cycle distribution. Subconfluent cultures of LoVo or HT29 cells were treated with either DMSO alone or various doses of GSE (0-100 μg/mL). After 12, 24, and 48 hours of treatment times, cells were harvested by brief trypsinization and centrifugation. Cell pellets were washed twice with ice-cold PBS, and ~0.5 × 10^6 cells were suspended in 500 μL of saponin/propidium iodide solution [0.3% (w/v) saponin, 25 μg/mL propidium iodide, 0.1 mmol/L EDTA, and 10 μg/mL RNase A in PBS] and incubated at 4°C for 24 hours in the dark. Stained cells were analyzed by flow cytometry analysis at FACScan core services of the University of Colorado Cancer Center (Denver, CO).

Quantitative apoptotic cell death assay. To quantitate GSE-induced apoptotic death of human colorectal cancer cells (LoVo and HT29), Annexin V and propidium iodide staining was done using Vybrant Apoptosis Assay kit 2 essentially as described in manufacturer's protocol. Briefly, after treatment (DMSO vehicle control, 25, 50, and 100 μg/mL doses of GSE for 24 hours), cells were harvested by brief trypsinization and centrifugation. After two washes with ice-cold PBS, cells were stained following protocol provided within the kit. The cells were then subjected to flow cytometric analysis.

Western immunoblotting. HT29 and LoVo cells, at 60% confluency, were treated with DMSO alone or different concentrations of GSE (0-100 μg/mL in DMSO) for different time points (12-48 hours). At the end of each treatment, cell lysates were prepared in nondenaturing lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethyl-sulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP40, 5 units/mL aprotinin] as published earlier (14). Cytoplasmic and nuclear extracts were prepared as described earlier (14). Immunoblot analysis using equal amount of protein lysate per sample was done as described earlier (14). Membranes were probed with desired primary antibodies followed by peroxidase-conjugated appropriate secondary antibody and visualized by enhanced chemiluminescence detection system.

HT29 tumor xenograft study. To study the *in vivo* efficacy of GSE against human colon carcinoma HT29 tumor xenograft growth, three million HT29 cells mixed in Matrigel were s.c. injected on the right flank of each athymic male *nu/nu* mice (National Cancer Institute, Frederick, MD). Animal care and treatments were in accord with Institutional guidelines and approved protocol. After 24 hours, mice were randomly divided in two groups (n = 9 mice per group) and gavaged with sterile saline (control group) or 200 mg/kg dose of GSE in sterile saline (treatment group) for 5 days/wk for 8 weeks. Tumor volume, body weight, and diet consumption were monitored weekly during the entire experiment. At the end of the treatment, tumors were collected, weighed, and stored at −80°C freezer for further analysis. Parts of the five randomly selected tumors from each group were homogenized in lysis buffer, and the resulting total cell lysates were analyzed by Western immunoblotting as described earlier (13).

Immunohistochemical detection of PCNA in tumors. Tumor samples were fixed in 10% buffered formalin for 12 hours and processed conventionally. Tumor sections were incubated with mouse monoclonal anti-PCNA antibody IgG2a (1:400) followed by biotinylated rabbit anti-mouse antibody IgG (1:200 in 10% normal rabbit serum) and conjugated horseradish peroxidase streptavidin and 3,3′-diaminobenzidine as published earlier (16). Finally, proliferating cells were quantified by counting the PCNA-positive cells and the total number of cells at 10 randomly selected fields at ×400 magnification in each tumor sample. The proliferation index was determined as (number of PCNA-positive cells × 100) / total number of cells.

In situ *apoptosis detection by terminal deoxynucleotidyl transferase–mediated nick-end labeling staining.* Tumor sections (those used for PCNA staining) were subjected to terminal deoxynucleotidyl transferase–mediated nick-end labeling staining using Tumor TACS *In situ* Apoptosis Detection kit as published recently (16). The apoptosis was

evaluated by counting the positive cells (brown stained) as well as the total number of cells at 10 randomly selected fields at ×400 magnification in each tumor sample. The apoptotic index was calculated as (number of apoptotic cells × 100) / total number of cells.

Immunohistochemical analysis of tumors for cyclin D1 expression. Cyclin D1 staining procedure was similar to PCNA staining using specific antibody for cyclin D1. Briefly, tumor sections were incubated overnight with rabbit polyclonal anti-cyclin D1 antibody in 1:400 dilutions followed by biotinylated anti-rabbit secondary antibody (Santa Cruz Biotechnology) and streptavidin-conjugated horseradish peroxidase and 3,3′-diaminobenzidine. Cyclin D1 immunoreactivity was analyzed in 10 randomly selected fields for each sample and scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), and 4+ (very strong staining). Mean immunoreactivity score was used for comparison.

Statistical analysis. All the *in vitro* study results shown are representative of at least two to three independent experiments. In cell culture studies, statistical significance of differences between control and GSE-treated samples were calculated by Student's *t* test (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA). Bands were scanned with Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA), and the mean density of each band was analyzed by the ScionImage program (NIH, Bethesda, MD). Densitometric data presented below bands are fold change compared with control and adjusted with β-actin for each treatment time. All the microscopic immunohistochemical analyses were done by Zeiss Axioscop 2 microscope (Carl Zeiss, Inc., Jena, Germany). Quantitative data are shown as mean and SE. In xenograft study, the statistical significance of difference between control and GSE-treated group was determined by ANOVA followed by Bonferrani *t* test. In each case, *P*s < 0.05 were considered statistically significant.

Results

GSE inhibits growth and induces death in human colorectal cancer cells. Our first aim was to examine the effect of GSE on the growth of HT29 and LoVo human colorectal cancer cell lines. In HT29 cells, GSE treatment at 50 and 100 μg/mL concentrations decreased total cell number by 36% to 43% ($P < 0.05\text{-}0.01$) and 23% to 79% ($P < 0.01\text{-}0.001$) after 24 and 48 hours of treatments, respectively (Fig. 1A). However, GSE treatments for 12 hours or the lowest concentration of GSE (25 μg/mL) used in the study up to 48 hours did not show any effect on cell growth. The observed growth inhibitory effect of GSE at 50 and 100 μg/mL concentrations was accompanied by a decrease in live cell number (38-85%, $P < 0.01\text{-}0.001$; data not shown). Contrary to the insignificant effect of 100 μg/mL concentration of GSE on cell growth in 12 hours of treatment, a significant increase in cell death (3% in control versus 12% in GSE treatment, $P < 0.01$) was observed (Fig. 1B). The higher concentrations of GSE showed dose- and time-dependent increase (up to 33%, $P < 0.001$) in cell death after 24 and 48 hours of treatments (Fig. 1B).

In LoVo cells, GSE inhibited cell growth in a dose- and a time-dependent manner. GSE (25, 50, and 100 μg/mL) treatments for 12 hours decreased total cell number by 11%, 27%, and 48% ($P < 0.001$), respectively, which was further decreased with the increase in the treatment time (13-58% at 24 hours and 20-85% at 48 hours, $P < 0.01\text{-}0.001$; Fig. 1C). A
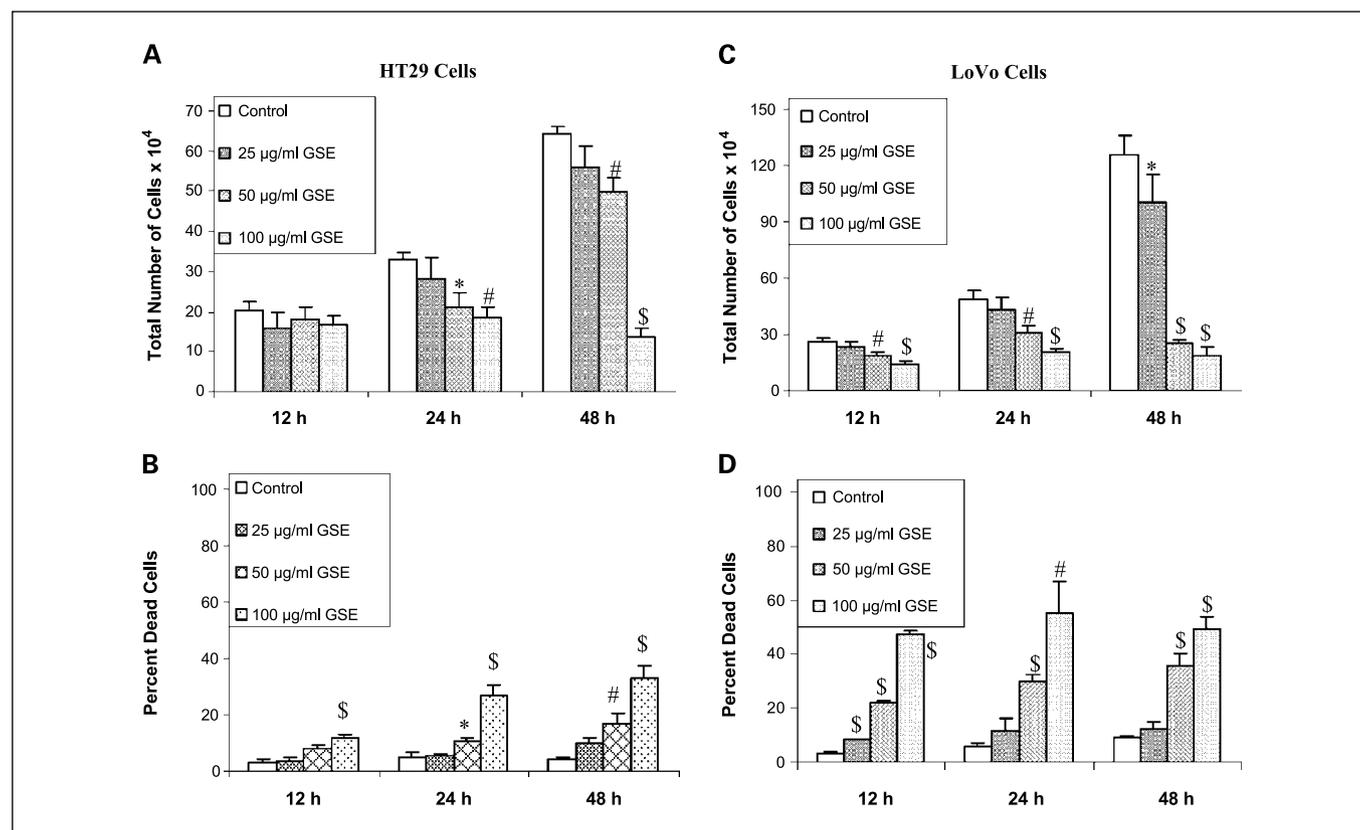
Fig. 1. GSE inhibits the growth of human colorectal cancer HT29 and LoVo cells. Cells were plated at a density of 5,000/cm² overnight and treated with either DMSO alone or GSE at the concentrations of 25 to 100 μg/mL in DMSO. After 12, 24, and 48 hors of these treatments, cells were collected after brief trypsinization and counted with hemocytometer after trypan blue staining. Columns, mean total cell number (*A* and *C*), and cell death (*B* and *D*) of three independent samples for each treatment; bars, SD. *, *P* ‹ 0.05; #, *P* ‹ 0.01; and $, *P* ‹ 0.001 versus control.
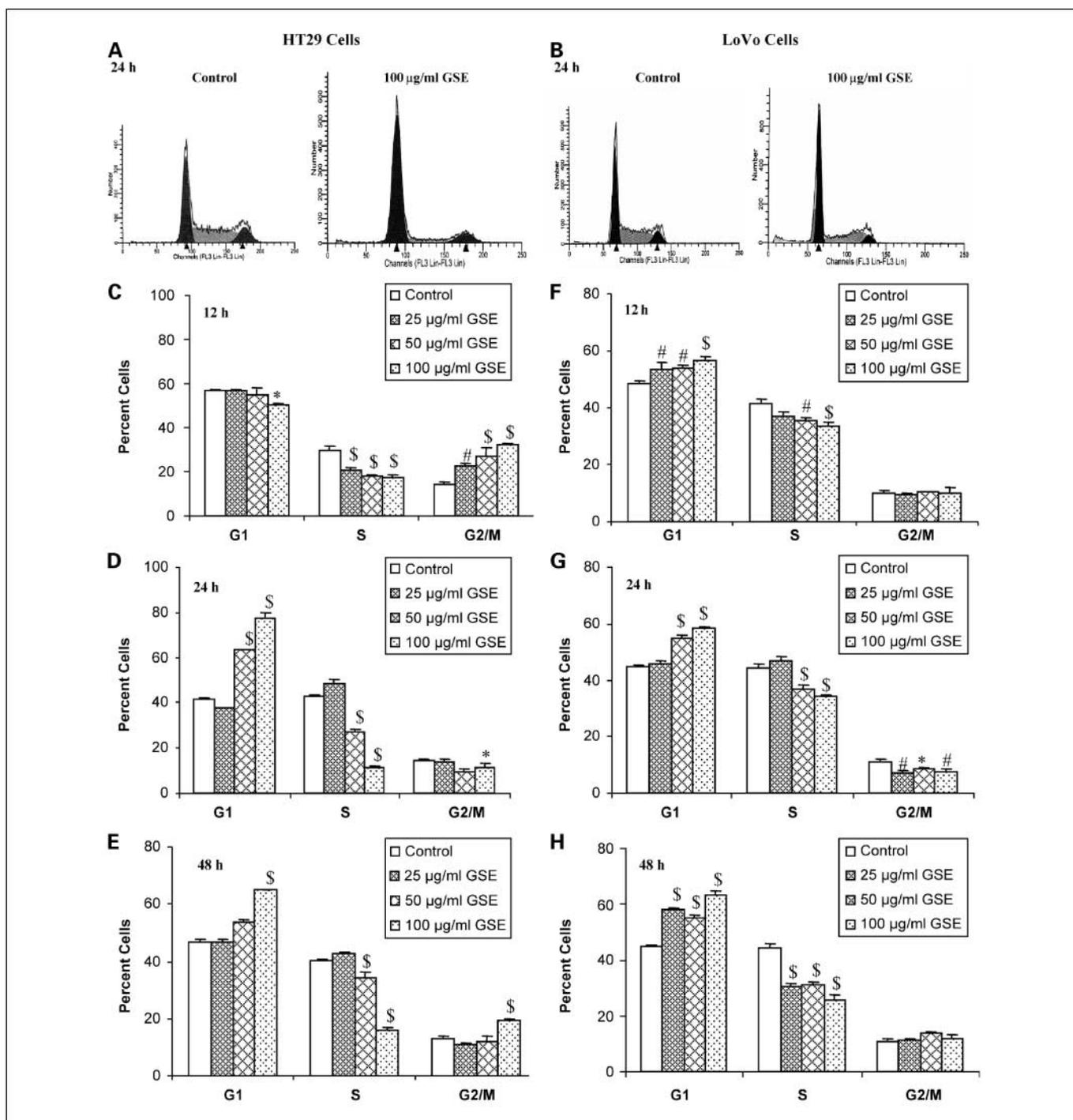
Fig. 2. GSE induces cell cycle arrest in human colorectal cancer HT29 and LoVo cells. Cells were treated with either DMSO alone (control) or varying concentrations of GSE (25-100 μg/mL) for 12, 24, and 48 hours. At the end of these treatments, cells were collected and incubated with saponin/propidium iodide solution at 4°C for 24 hours in dark and subjected to fluorescence-activated cell sorting analysis as detailed in Materials and Methods. Representative fluorescence-activated cell sorting scans for HT29 cells (*A*) and LoVo cells (*B*). Quantitative cell cycle distribution data for HT29 cells (*C-E*) and LoVo cells (*F-H*) are shown for 12, 24, and 48 hours of GSE treatment, respectively. Columns, mean of three independent plates; bars, SD. *, *P* < 0.05; #, *P* < 0.01; and $, *P* < 0.001 versus control.

corresponding decrease in live cell number was also observed, which accounted for 17% to 72%, 18% to 80%, and 23% to 92% (*P* < 0.001) following GSE (25-100 μg/mL) treatments for 12, 24, and 48 hours, respectively (data not shown). Concomitantly, there was a significant dose-dependent increase in cell death from 3% to 9% in controls to 8% to 55% in GSE treatments (*P* < 0.05 at lowest concentration, *P* <

0.01-0.001 at two higher concentrations) under similar condition (Fig. 1D).

GSE induces cell cycle arrest in human colorectal cancer cells. Based on the cell growth inhibitory effect of GSE, its effect on cell cycle progression in colorectal cancer cells was anticipated, and therefore, a cell cycle distribution analysis was done. The representative flow cytometry scans for control and
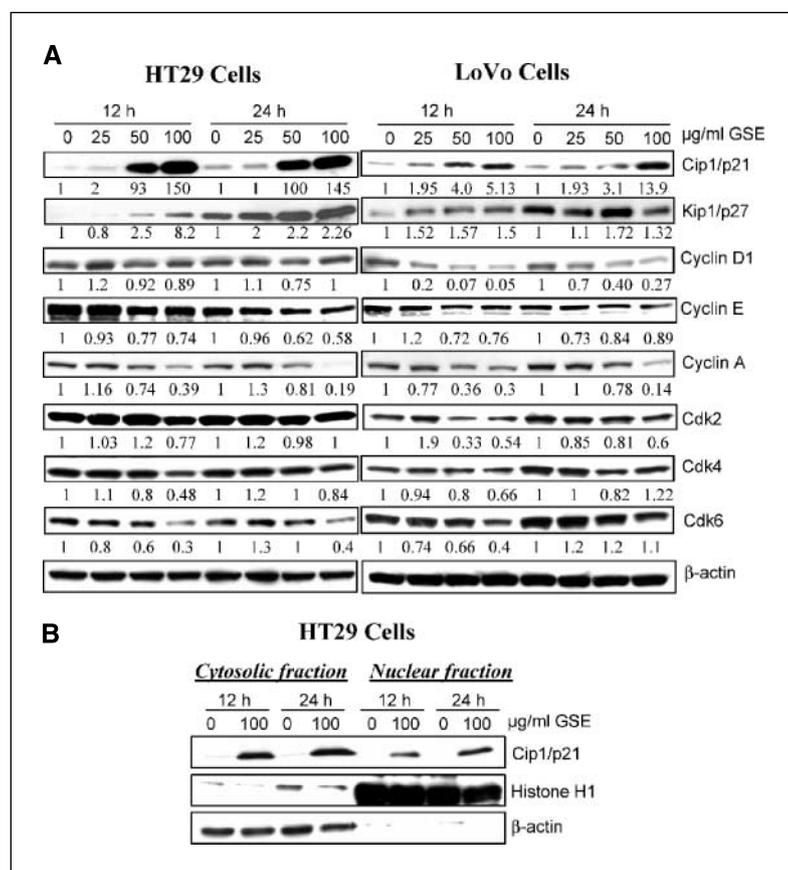
Fig. 3. GSE modulates the protein levels of cell cycle regulatory molecules in HT29 and LoVo cells. *A,* cells were treated with either DMSO alone (control) or varying concentrations of GSE (25-100 μg/mL). At the end of the treatments, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting. Membranes were probed with specific primary antibodies for Cip1/p21, Kip1/p27, cyclin D1, cyclin E, cyclin A, CDK2, CDK4, and CDK6 followed by peroxidase-conjugated appropriate secondary antibodies and visualized by enhanced chemiluminescence detection system. Membranes were stripped and reprobed with anti-β-actin antibody for loading control. Representative blot. Densitometric data are shown as fold change versus control below each band. *B,* cells were treated with either DMSO alone (control) or 100 μg/mL GSE, and cytoplasmic and nuclear fractions were prepared and analyzed for Cip1/p21 protein level as described in Materials and Methods. Membrane was stripped and reprobed for histone H1 and β-actin for loading correction and checking the purity of the fractions.

100 μg/mL GSE-treated HT29 and LoVo cells for 24 hours are shown in Fig. 2A and B that clearly indicate an increase in counts for G_1 phase cell population. In HT29 cells, GSE treatment for 12 hours led to a significant and dose-dependent accumulation of cells at G_2-M phase (Fig. 2C). However, at later treatment times (24 and 48 hours), a strong G_1 arrest was observed at 50 and 100 μg/mL concentrations of GSE (Fig. 2D and E). In LoVo cells, GSE treatment (25-100 μg/mL) led to a significant dose-dependent accumulation of cells in G_1 phase ($P < 0.01$-0.001) as early as 12 hours of the treatment, and this effect remained sustained even after 48 hours (Fig. 2F-H). In each case, GSE decreased S phase cell population. Overall, the observed cell cycle arrest (predominantly in G_1 phase) by GSE possibly accounts for its cell growth inhibitory effect in colorectal cancer cells.

GSE modulates the protein levels of CDKIs, CDKs, and cyclins in human colorectal cancer cells. Because GSE showed strong cell cycle arrest at G_1 phase in both colorectal cancer cell lines, we next studied the effect of GSE on G_1 phase cell cycle regulators. Immunoblot analysis revealed a strong dose-dependent increase in Cip1/p21 protein level (up to 150-fold) by GSE, which was evident after 12 hours of the treatment (Fig. 3A) and persisted until 48 hours (data not shown) in HT29 cells. A similar dose-dependent increase in Cip1/p21 (up to 14-fold) was also observed in LoVo cells following identical GSE treatments, but the effect was not as strong as in HT29 cells (Fig. 3A). GSE also increased Kip1/p27 protein levels (up to 8-fold) in HT29 cells; however, this effect was again moderate in LoVo cells (up to 2-fold; Fig. 3A). In LoVo cells, 24 hours of GSE treatment at 25 and 50 μg/mL concentrations showed a moderate increase in Kip1/p27 protein level; however, this

trend declined at 100 μg/mL concentration of GSE (Fig. 3A). GSE treatments also caused a moderate to strong dose-dependent decrease in the protein levels of cyclin D1, cyclin E, cyclin A, CDK4, and CDK6 in both the cell lines, without any considerable effect on CDK2 protein levels (Fig. 3A). In HT29 cells, a moderate decrease in cyclin B1 by highest dose of GSE (100 μg/mL) was observed (data not shown) and was associated with the moderate increase in G_2-M arrest. Because a prominent effect of GSE was observed on Cip1/p21 induction, its cytoplasmic and nuclear localization was also examined in HT29 cells. Consistently, an increase in both cytoplasmic and nuclear levels of Cip1/p21 protein was observed by 12 and 24 hours of GSE treatment (Fig. 3B).

GSE causes apoptotic cell death of human colorectal cancer cells. Because GSE treatments caused death of both HT29 and LoVo cells (Fig. 1), the possible apoptotic effect of GSE on these colorectal cancer cells was next examined by Annexin V and propidium iodide staining, where cells were treated with GSE (25-100 μg/mL) for 24 hours under similar conditions as in cell growth studies. GSE treatment at 50 and 100 μg/mL concentrations showed a significant dose-dependent increase (4- to 9-fold, $P < 0.001$) in apoptotic cell population in HT29 cells (Fig. 4A). The lowest GSE concentration did not show any apoptotic effect in HT29 cells. In LoVo cells, all three GSE concentrations (25, 50, and 100 μg/mL) caused a dose-dependent increase in apoptotic cells; however, it was significant only for the two higher concentrations of GSE (3- to 4-fold, $P < 0.001$; Fig. 4B). Based on these observations, next, we studied the effect of GSE on caspase-3 and PARP cleavage as the molecular markers of apoptosis induction. In this analysis,

we also included the 12 hours of GSE treatment time to assess its early effect on these molecular markers. GSE caused mostly a dose- and time-dependent increase in the cleaved levels of caspase-3 and PARP in both the cell lines (Fig. 4C). In our experimental condition, cleaved caspase-3 was not detectable after 12 hours of GSE treatments in HT29 cells.

GSE feeding inhibits HT29 colon carcinoma tumor xenograft growth in athymic nude mice. In vivo efficacy of GSE against human colon carcinoma was studied in tumor xenograft in athymic mice by ectopic implantation of HT29 cells. Oral gavage feeding of GSE at 200 mg/kg dose, 5 days/wk did not show any considerable change in body weight (Fig. 5A) and diet consumption (Fig. 5B) during the 8 weeks of experiment. Furthermore, we also did not observe any adverse health effect as monitored by activity and posture of mice. At the end of the experiment, GSE reduced tumor volume per mouse by 44% ($1,860.4 \pm 284.4$ mm^3 in control versus $1,038.2 \pm 134.0$ mm^3 in GSE-fed group; $P = 0.041$, one-way ANOVA, Bonferroni t test; Fig. 5C), although a time-dependent growth inhibitory effect of GSE on tumor growth was evidenced throughout the study. The decrease in tumor growth rate from week 5 to week 6 in control group is because three mice were euthanized in this group at week 5 due to the large tumor size beyond which it was not permissible to keep these mice in the protocol as per the institutional animal protocol guidelines. At the end of the experiment, tumor weight was also found decreased by 33% ($P = 0.005$) in GSE-treated group of mice (Fig. 5D). These results suggest an in vivo anticancer efficacy of GSE without any toxicity in nude mice HT29 colorectal cancer xenograft model.

In vivo *antiproliferative effect of GSE in HT29 tumor xenografts.* To assess the in vivo effect of GSE feeding to mice on its antiproliferative response associated with the inhibition of tumor xenograft growth, tumor samples were immunohistochemically analyzed for PCNA staining. Qualitative microscopic examination of PCNA-stained tumor sections showed substantial decrease in PCNA-positive cells in GSE-fed group of tumors compared with control group of tumors (data not shown). The quantification of PCNA immunohistochemical staining showed $16 \pm 2\%$ PCNA-positive cells in GSE-fed group of tumors compared with $31 \pm 3\%$ in control, accounting for a 48% ($P < 0.001$) decrease in proliferation index (Fig. 6A). Tumors sections

were also immunohistochemically analyzed for cyclin D1 protein expression, in which GSE showed a moderate but significant decrease (15%, $P = 0.01$) in cyclin D1 immunoreactivity in tumors (Fig. 6B). These findings were in accord with in vitro observations. Because in cell culture study, Cip1/p21 upregulation was identified as a potential mechanism for inducing cell cycle arrest to inhibit proliferation in colorectal cancer cells, HT29 tumor xenograft lysates were also examined for Cip1/p21 protein level by Western immunoblotting. Similar to the finding in cell culture study, GSE feeding to mice increased the Cip1/p21 protein level by $\sim$2-fold ($P = 0.041$) in HT29 tumor xenograft (Fig. 6C). Overall, these findings suggest Cip1/p21 as a potential target for GSE efficacy against colorectal cancer in both cell culture and tumor xenograft models.

In vivo *proapoptotic effect of GSE in HT29 tumor xenografts.* In vivo apoptotic response of GSE feeding in HT29 tumor xenografts was investigated by terminal deoxynucleotidyl transferase–mediated nick-end labeling staining. Microscopic examination of the tumor sections showed an increase in terminal deoxynucleotidyl transferase–mediated nick-end labeling–positive cells in GSE-fed group of tumors compared with control group of tumors (data not shown). The quantitative evaluation of apoptosis in tumors showed that 200 mg/kg dose of GSE causes $28 \pm 2\%$ apoptotic cells compared with $18 \pm 2\%$ in control, which accounted for 1.6-fold ($P = 0.002$) increase over that of control (Fig. 6D). TACS-nuclease was used to generate DNA fragments with free 3'-OH end and showed positive staining in all the nuclei (positive control), whereas labeling buffer was used instead of TdT that did not show any considerable positive staining (negative control; data not shown). To further confirm the in vivo apoptotic effect of GSE, tumor lysates were analyzed for the cleaved levels of PARP. As expected, the cleaved levels of PARP were mostly higher in GSE-fed group of tumors (Fig. 6E). Overall, these findings translate the in vitro apoptotic effect of GSE in to in vivo condition in colorectal cancer cells.

Discussion

The main objective of present study was to evaluate anticancer efficacy and associated mechanisms of GSE in human colorectal

Fig. 4. GSE causes apoptotic death of HT29 and LoVo cells. Cells were treated with DMSO (control) or different concentrations of GSE (25-100 μg/mL) for 24 hours. At the end of the treatment, cells were collected and stained with Annexin V-propidium iodide and analyzed by flow cytometry. Columns, mean % apoptotic cells in HT29 cells (*A*) and LoVo cells (*B*) of three independent samples for each treatment; bars, SD. *, $P < 0.05$; #, $P < 0.01$; and $, $P < 0.001$ versus control. *C,* HT29 and LoVo cells were treated with either DMSO alone (control) or varying concentrations of GSE (25-100 μg/mL) for 12 and 24 hours, and cell lysates were analyzed for cleaved caspase-3 and PARP levels by Western immunoblotting as described in Materials and Methods. Membranes were stripped and reprobed with anti-β-actin antibody for loading control. Representative blot.
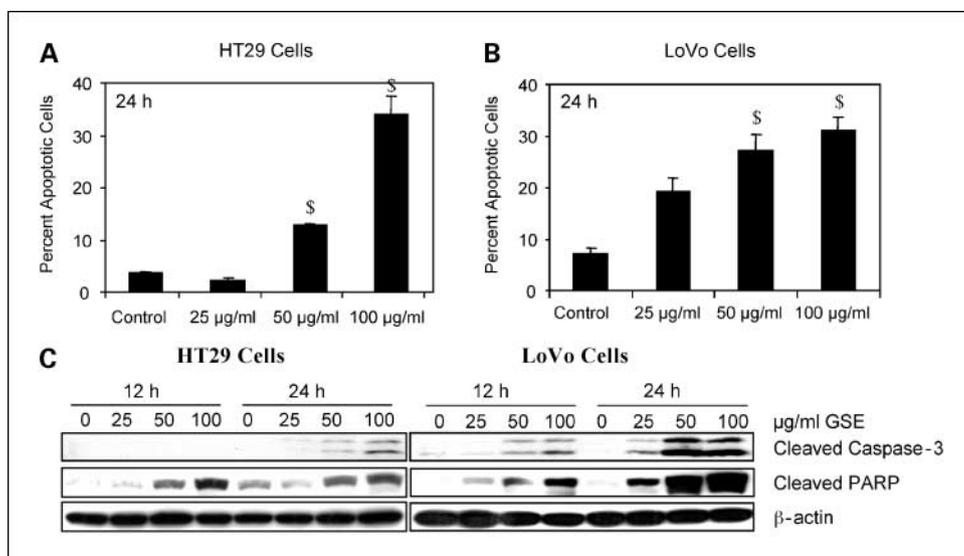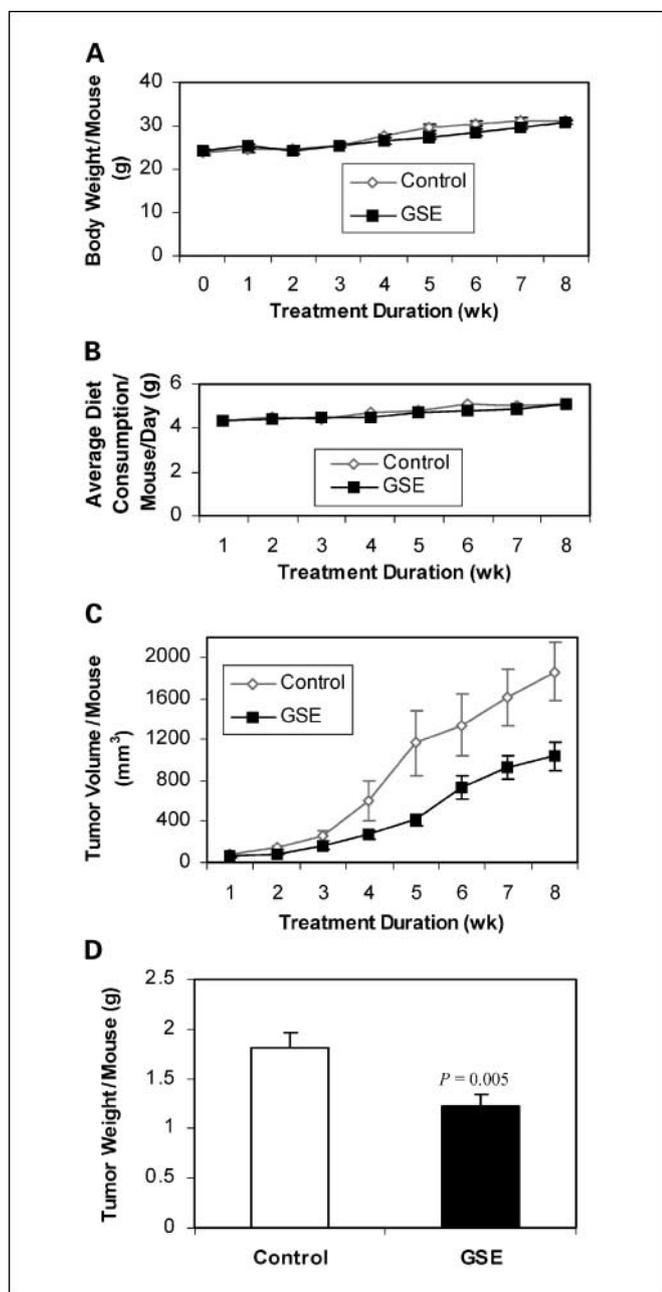
Fig. 5. GSE feeding inhibits HT29 tumor xenograft growth in athymic nude mice. Each mouse was ectopically implanted with three million HT29 cells mixed in Matrigel on the right flank. After 24 hours, mice were gavaged with saline (control group) or 200 mg/kg dose of GSE in saline for 5 days/wk for 8 weeks: (*A*) mean body weight/mouse (g) and (*B*) average diet consumption per mouse per day (g) are plotted as a function of week of GSE treatment. *C,* tumor volume/mouse (mm^3) as a function of GSE treatment. *D,* tumors were harvested at the end of the experiment and weighed (tumor weight per mouse, g). Points/columns, mean of eight to nine mice in each group; bars, SE (*A, C,* and *D*). Statistical significance of difference between control and GSE-fed groups was calculated by one-way ANOVA followed by Bonferroni *t* test.

cancer cells in cell culture and to translate the *in vitro* findings in to an *in vivo* preclinical colorectal cancer model. Our study revealed that GSE causes cell growth inhibition via cell cycle arrest specifically in G$_1$ phase and induces apoptosis in human colorectal cancer HT29 and LoVo cells in culture studies. In these studies, strong up-regulation of Cip1/p21 expression and

caspase activation were also noted as plausible mechanisms of the observed GSE effects in these colorectal cancer cells. In HT29 tumor xenograft study, oral feeding of GSE inhibited tumor growth, which was accompanied with antiproliferative and proapoptotic effects together with an increased expression of Cip1/p21 protein and PARP cleavage.
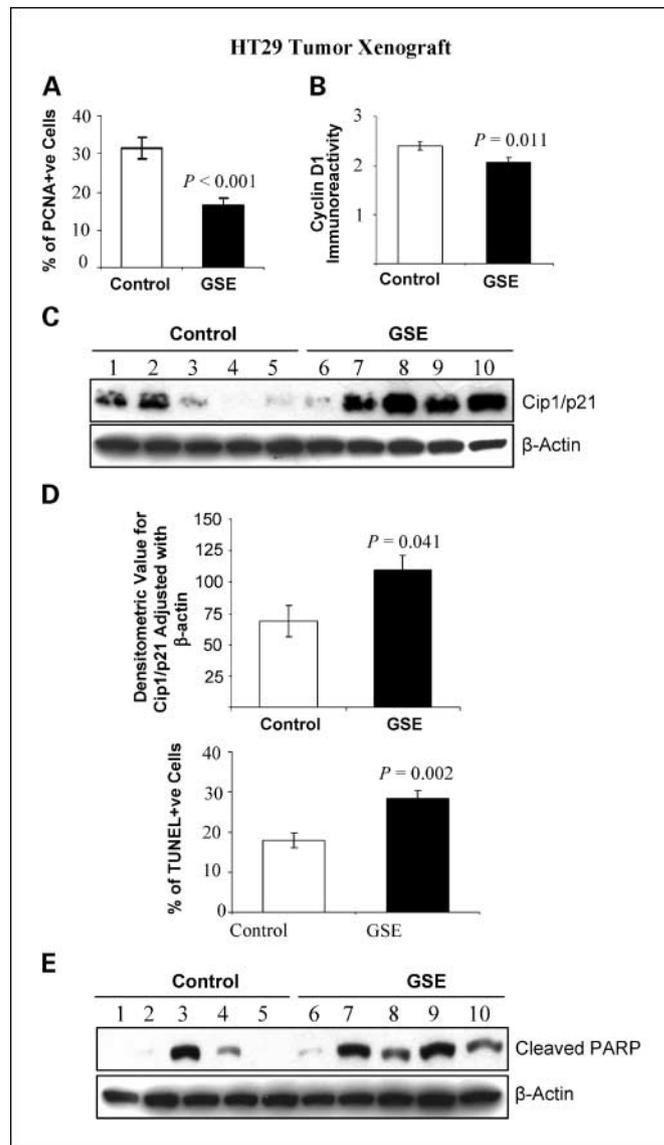

Fig. 6. GSE feeding inhibits cell proliferation and induces apoptosis in HT29 tumor xenograft. The tumor xenograft collected at the end of the study in Fig. 5 were analyzed by immunohistochemistry and immunoblotting for the molecular biomarkers and events associated with proliferation and apoptosis as described in Materials and Methods. Immunohistochemical staining of tumors was quantified for (*A*) PCNA-positive cells for proliferation index, (*B*) cyclin D1 immunoreactivity score, and (*D*) terminal deoxynucleotidyl transferase – mediated nick-end labeling – positive cells for apoptotic index. *C* and *E,* parts of the five randomly selected tumors each from five individual mouse in control (*lanes 1-5*) and GSE-fed (*lanes 6-10*) groups were used for total cell lysate preparation and analyzed by Western immunoblotting for (*C*) Cip1/p21 expression and (*E*) cleaved PARP as described in Materials and Methods. Membranes were stripped and reprobed with anti-β-actin antibody for loading control. Representative blot. Densitometric analysis was done for Cip1/p21. Columns, mean (calibrated with β-actin) of five individual tumor samples; bars, SE. All immunohistochemical data are mean ± SE of eight to nine samples for each group. Statistical significance of difference between control and GSE-fed groups was calculated by one-way ANOVA followed by Bonferroni *t* test.

Here, it should be noted that p53 is nonfunctional (due to point mutation) in HT29 cells, which represents a relatively advance stage of colorectal cancer compared with LoVo cells having functional p53 (wild type; ref. 31). Our findings showed that GSE strongly inhibited cell growth in both the colorectal cancer cell lines; however, with the increase in treatment time lower concentrations of GSE (25 and 50 μg/mL) became relatively more effective in LoVo cells than in HT29 cells. GSE-induced cell growth inhibition was accompanied by a strong induction of G_1 arrest in both the cell lines; however, GSE treatment of HT29 cells also showed G_2-M arrest, which was sustained at the highest dose of GSE (100 μg/mL) even after 48 hours of the treatment. It seems that the differential effect of GSE on cell cycle progression is associated with the p53 status in the colorectal cancer cells; however, it would be interesting to further investigate and define the role of p53 in cell cycle effects of GSE in colorectal cancer cells.

CDKs, CDKIs, and cyclins are the key regulatory molecules in cell cycle progression (27, 32). The progression through various phases of cell cycle is governed by sequential activation/inactivation of different CDKs, which is mediated by their interaction with activating partners (cyclins) or inactivating partners (CDKIs; refs. 27, 32). The association of CDK4/CDK6 with D-type cyclins regulates the G_1 phase progression, whereas progression through G_1-S transition is primarily regulated by CDK2-cyclin E/A complex (33). In the present study, GSE decreased protein levels of CDK4, CDK6, cyclin D1, and cyclin A with comparatively less decreasing effect on CDK2 and cyclin E in both colorectal cancer cell lines. GSE strongly increased Cip1/p21 protein level in both colorectal cancer cell lines, irrespective of p53 status indicating its p53-independent effect on Cip1/p21 induction in HT29 cells. Although, Cip1/p21 is transcriptionally up-regulated by p53 through its binding to an upstream regulatory site of the *Cip1/p21* gene (34), the transactivation of Cip1/p21 could also occur by p53-independent mechanisms by a variety of stimuli, such as transforming growth factor-β, fibroblast growth factor, platelet-derived growth factor, okadaic acid, or cycloheximide (35–37). Furthermore, Kip1/p27 protein level was also increased by GSE with relatively stronger effect in HT29 cells compared with LoVo cells. In this regard, Kip1/p27 is shown to be up-regulated in response to antiproliferative signals (38). Overall, these findings favor the decrease in G_1 phase CDK-cyclin activity, in part via their likely increased interaction with CDKIs, leading to a G_1 phase arrest in cell cycle progression by GSE as observed in the present study.

The loss of apoptotic function is a major contributor to resistance of cancer cells to cytotoxic chemotherapeutic agents (39–41). Induction of apoptosis under such circumstances is highly desirable. In this regard, GSE decreased colorectal cancer cell survival via an induction of programmed cell death involving caspase activation, which identifies yet another mechanism (apart from cell cycle arrest) of GSE for its anticancer activity against colorectal cancer. Consistent with the cell cycle effect and Cip1/p21 up-regulation, GSE also induced apoptosis in both the colorectal cancer cell lines irrespective of their p53 status. Additional mechanistic studies, however, are required in future to elucidate whether Cip1/p21 has a role in GSE-induced apoptosis in colorectal cancer cells.

Based on the encouraging *in vitro* anticancer efficacy of GSE against colorectal cancer, we further studied its efficacy in a preclinical animal model by ectopic implantation of HT29 xenograft in athymic nude mice. The findings in this study provide an *in vivo* evidence for the efficacy of oral GSE against advanced colorectal cancer growth without any toxicity in nude mice. Immunohistochemical analysis of tumors for the end point biomarkers, such as cell proliferation and apoptosis, showed inhibition of tumor cell proliferation and an induction of apoptotic cell death in GSE-fed group of tumors. Furthermore, consistent with the findings in cell culture, an increase in Cip1/p21 protein expression in GSE-fed group of tumors was also observed, and these tumors showed an increased level of cleaved PARP, indicating the effect of GSE on caspase activation. Overall, our present data for PCNA, cyclin D1, and Cip/p21 show that GSE has a strong and significant *in vivo* antiproliferative effect against colorectal cancer growth, and that these molecular alterations by GSE possibly lead to a cell cycle arrest as well as an induction of apoptotic cell death.

In summary, our results show that GSE inhibits cell growth and induces cell cycle arrest and apoptosis in human colorectal cancer cells and modulates cell cycle regulators with a strong effect for Cip1/p21 up-regulation. Usually, p53 plays a regulatory role in Cip1/p21 induction; however, in our studies, GSE up-regulates Cip1/p21 independent of p53 because HT29 cells showing a robust increase in Cip1/p21 harbor nonfunctional p53, although LoVo cells carry wild-type p53. Therefore, it would be of significance to investigate in future studies the p53-independent mechanisms of Cip1/p21 induction by GSE that might have a wide implication in cancer chemoprevention as p53 inactivation is one of the primary events in initiation, growth and progression of many types of cancers, including colorectal cancer. Furthermore, findings in xenograft study translate the anticancer effects and associated mechanisms of GSE observed in cell culture experiments in to an *in vivo* preclinical colorectal cancer model. However, a dose-dependent *in vivo* study with GSE is needed in future that would provide additional information regarding the lowest effective as well as highest nontoxic doses of GSE, which would be useful for the translational studies.

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