ORIGINAL ARTICLE

Antiproliferative and apoptotic effects of Grape Seed Extract on human colon cancer cell line HCT116

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ABSTRACT

Grape seed extract (GSE) has been proven to inhibit cell proliferation and induce apoptosis in various human carcinoma cell lines. Herein, we evaluated the in vitro antiproliferative and apoptotic effects of GSE against colon cancer cell line HCT116. The cytotoxic effect of GSE was examined on human colon cancer HCT116 and normal WISH cells. In addition, we evaluated the antiproliferative and apoptotic effects of different concentrations of GSE (25, 50 and 100µg/ml) at different sampling time (24, 48, 72 and 96hrs) on HCT116 cells using cell proliferation and DNA fragmentation assays respectively. Because the p53 tumor suppressor protein plays an important role in cellular proliferation and apoptosis, we examined p53 mRNAs expression in relation with BAX and BCL-2 mRNAs expression. The present study revealed that GSE did not suppress the proliferation of normal human WISH cells, while, GSE suppressed proliferation and induced apoptosis in human HCT116 colon cancer cells indicating that GSE preferentially target cancer cells while sparing their normal counterparts. GSE exerts its effect on HCT116 cell line via p53, BAX upregulation and BCL-2 downregulation especially at 72-96hrs in a dose-dependent manner. Taken together, our findings suggest that GSE could be an effective complementary medicine agent against colorectal cancer possibly due to its strong growth inhibitory and apoptosis inducing effects.

Key words: Colorectal cancer; HCT116; WISH; GSE; P53; BCL-2

Introduction

Colorectal cancer (CRC) is the third most common cancer in both men and women worldwide; the overall mortality due to this malignancy accounts for 10% of all the cancer-associated deaths. CRC is one of the disease conditions where dietary habits and life style are major etiological factors. Diets rich in fat, animal proteins, and low in fiber are often considered risk factors for developing CRC, whereas those rich in fruits, vegetables, and whole grains are often recommended for reducing the risk of this malignancy (Campos et al., 2005).

Fruits and vegetables have been hypothesized to be major dietary contributors to cancer prevention because they are potentially full of anticancer substances (Glade, 1997). Consequently, in recent years some studies attempted to isolate and characterize potential chemopreventive agents present in fruits and vegetables. In this regard, many phytochemicals of different chemical nature, such as catechins, bioflavonoids, proanthocyanidins and phyto-estrogens, have revealed promising chemopreventive and/or anticancer efficacy in several cell cultures and animal models (Faria et al., 2006; Liu et al., 2010).

Vitis vinifera (Grape) is a rich source of several biologically active compounds including anthocyanins, proanthocyanidins, and stilbenes (Nassiri-Asl and H. Hosseinzadeh, 2009). Grape seed extract (GSE), a mixture containing about 95% standardized proanthocyanidins, is a popular dietary supplement due to its anti-cancer and anti-inflammatory properties (Agarwal et al., 2002). In vitro studies showed that GSE has significant growth inhibitory action on a variety of colon cancer cells in a dose- and time-dependent manner (Kaur et al., 2008). GSE significantly inhibited cell viability and elevated apoptosis in cancer cells without altering the viability of the normal colon cell lines, thus selectively targeting cancer cells (Laurent et al., 2004). GSE induced G1 phase arrest and caspase-3 mediated apoptosis in cancer cells (Laurent et al., 2004; Kaur et al., 2006). GSE might thus exert its beneficial effects by elevating apoptosis and suppressing proliferative pathways. Earlier reports suggest that GSE obtained from different commercial vendors can produce comparable biological effects via cell growth suppression in a panel of human colon cancer cell lines (Kaur et al., 2008) indicating that minor differences in proanthocyanin content and composition of GSE may not be very important.
Recently, it has become clear that a variety of anticancer agents can induce programmed cell death or apoptosis. The apoptotic process is modulated by various tumor suppressor genes including p53 and proto-oncogenes (Thompson, 1995). It has been assumed that the presence of wild-type p53 is essential for apoptosis (Strasser et al., 1994). The activation of p53 in the cells leads to either DNA repair and recovery or apoptosis (Kuerbitz et al., 1992) and p53 has also been shown to have an effect on apoptosis by regulating BCL-2 and BAX expression (Kobayashi et al., 1997). BCL-2, a proto-oncogene originally discovered in a follicular B-cell lymphoma (Tsujimoto et al., 1985), can inhibit apoptosis via the regulation of reactive oxygen species (Kane et al., 1993). BAX, a homolog of BCL-2, has been shown to form heterodimers with BCL-2. It has been suggested that the ratio of BCL-2 to BAX may determine cell fate, survival or death, when exposed to apoptotic stimuli such as removal of the growth factor (Oltvai et al., 1993).

In the present study, we evaluated the cytotoxic effect of GSE on HCT116 human colon cancer cells and comparing its effect on WISH human normal epithelial cells. We estimated the antiproliferative and apoptotic effect of GSE on HCT116 colon cancer cells. Apoptosis was examined in the context of p53, BCL-2 and BAX mRNAs expression and DNA fragmentation assay.

Material And Methods

Grape seed extract (GSE):

GSE-standardized preparation, constituting of at least 95% (wt/wt) procyanidins (China, Xiamen FGS) was dissolved in water and incubated in a boiling water bath for 30 min. The solution was centrifuged at 2,000 rpm (400 xg) for 10 min to remove any insoluble ingredients (Lu et al., 2009) and the clear part was filtered through a 0.2-μm filter.

Cell culture:

The human colorectal cancer HCT116 and normal human epithelial WISH cell lines were purchased from tissue culture Lab in VACSERA Institute, Agoza, Egypt (The holding company for biological products and Vaccines). Cells of HCT116 and WISH were seeded into 75cm² flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 2mM L-glutamine and antibiotics (penicillin 100 IU/ml, streptomycin 100μg/ml). The cultures were kept under standard culture conditions (37°C, 95% humidified air and 5% CO2).

Cytotoxicity assay by Sulphorhodamine B (SRB) method:

The monolayer cell cultures of HCT116 and WISH were separately trypsinized and suspended in fresh medium at a cell density of 1.0 X 10⁵/ml and then plated in 96 well tissue culture plates using RPMI-1640 supplemented with 10% FBS. After 24 hours, when a monolayer was formed, the supernatant was flicked off; 100μl of serum free media was added directly to the cells containing serial dilutions of GSE (23.43-3000μg/ml). The plates were then incubated at 37°C for 72 hours in 5% CO₂ incubator and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, the supernatant was discarded and 150μl of 10% trichloroacetic acid (TCA) was added to the wells. The plates were incubated at +4°C for one hour. The plates were washed three times with distilled water to remove TCA completely from cells. Then the plates were stained with 70μl of 0.4% SRB in 1% acetic acid and incubated for 10 minutes at room temperature. The unbound dye was removed by rapidly washing many times with 1% acetic acid. The plates were then air-dried, 150μl of 10 mM Tris base PH 7.4 was added to the wells to solubilize the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using an ELISA microplate reader at a wavelength of 540nm (Patel et al., 2009).

The percentage growth inhibition was calculated using following formula and values were plotted by using GraphPad PRISM program (GraphPad, UK). Generated graphs were used to calculate the inhibitory concentration 50 (IC50).

\[
\text{% Growth inhibition} = 100-\frac{(At-Ab)}{(Ac-Ab)} \times 100
\]

Where At = mean optical density (OD) of individual test group
Ab = mean OD of blank
Ac = mean OD of control group (untreated cells)

Cell proliferation assay by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method:

HCT116 cells were cultured in 96 well culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at concentration of 1X10⁵ cells/ml in RPMI-1640 medium containing 10% FBS. After 24 hours, the culture medium was discarded and the cells were treated with different concentrations of GSE 25, 50 and
100µg/ml in serum free media. After 24, 48, 72 and 96 hours of incubation, the cells were washed twice with phosphate-buffered saline (PBS). 50µl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5mg/ml PBS) was added to each well. The cells were incubated at 37°C for 4 hours followed by addition of 50µl of DMSO to each well. Then the plates were shaken and cell proliferation was measured as reduction of MTT into formazan at wavelength 570nm using an ELISA microplate reader. The untreated cells were used as control. Control and samples were assayed in quadruplicate for each concentration and replicated three times (Radhakrishnan et al., 2011).

The absorbance values were converted into percentages of cell viability using the following formula and data were plotted by using Microsoft Excel program.

\[
\text{Cell viability (\%)} = \frac{\text{Mean OD of treated cells}}{\text{Mean OD of control (untreated cells)}} \times 100
\]

-Statistical Analysis:

Statistical significance of differences between control and treated samples were calculated by using two-tailed Student's t-test (Sigma Stat 2.0, Jandel Scientific). P values < 0.05 were considered statistically significant versus control. Each assay/observation represents a mean ± SD of three independent values.

Expression of BAX, BCL-2 and P53 genes: RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

A total of twelve 75cm² flasks of HCT116 cells were treated with 25, 50, and 100 µg/mL of GSE in serum free RPMI-1640 media. At the end of the respective sampling time (24, 48, 72 and 96 hrs), cells were harvested by brief trypsinization and centrifugation. After two washes with ice-cold PBS, cells were further used for RNA extraction and DNA fragmentation assays.

Total RNA was extracted with Trizol reagent by the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was followed immediately by PCR amplification in a single tube using a One-Step RT-PCR System (Takara, Japan) which contains 5µl of 10x one step RNA PCR buffer, 10µl of 25mM MgCl₂, 5µl of 10mM dNTP, 1µl of RNase inhibitor, 1µl of AMV RTase XL, 1µl of AMV-optimized Taq, 1µl of forward primer (20 pmole), 1µl of reverse primer (20 pmole), 1µl of total RNA (200ng), 24 µl of RNase free distilled water. The primer sequences used for amplification were as follows: GAPDH (housekeeping gene), forward 5’-GAAGGTGAAGGTCGGAGTCA-3’ and reverse 5’-GAAGATGTGTTGATGGGATTTC-3’; BAX, forward 5’-CCGCCCCTGAGACACAGAC-3’ and reverse 5’-CAGAAAACATGTCAGCTGCCA-3’; BCL-2, forward 5’-TCCGATCAGGAAACGCTAGT-3’ and reverse 5’-TGGGTCTCTCCTAAAGCAAGGC-3’ (Suzuki et al., 1999); P53, forward 5’-CAGCCAAGTCTGTGACTTGCACGTAC-3’ and reverse 5’-CTATGTCGAAAAGTGTTCCTGTCATC-3’ (Lai et al., 2012). Standard amplification parameters were used with the following processes: 50ºC for 30 min for reverse transcription, 94ºC for 2 min. for RTase inactivation. Followed by 35 cycles with denaturation at 94ºC for 30 s, annealing at 60ºC for 30 s, and extension at 72ºC for 1.5 min; followed by a final extension at 72ºC for 10 min. RT-PCR products were analyzed on 1.5% agarose gel in the presence of 1 mg/mL of ethidium bromide. The expected size of the RT-PCR product is 108bp long for BCL-2, 133bp for BAX, 292bp for P53 and 226 bp for GAPDH.

Qualitative DNA fragmentation assay by agarose gel electrophoresis:

Orderly fragmentation of DNA in the form of a ladder due to endonucleolytic attack is reportedly considered as a characteristic of apoptosis and DNA fragmentation is considered as one of the later steps in the apoptotic process. HCT116 Cells were suspended in 100µl lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA and 1% SDS, incubated with 50 µg/ml proteinase K at 56°C overnight, and then centrifuged at 10,000 rpm for 30 min. Soluble DNA in the resulting supernatant was precipitated with ethanol at -20°C, dissolved in sterile ddH₂O. Electrophoresis was carried out in a 1.5% agarose gel containing ethidium bromide. The gel was examined and photographed under UV light to visualize intra-nucleosomal DNA fragmentation (laddering), characteristic of apoptosis.

Results:

Cytotoxicity assay of cultured cells:

The Cytotoxicity of GSE was evaluated by SRB assay and the half maximal inhibitory concentration IC50 values were derived from the dose-response curves (Fig. 1 and Fig. 2). The half maximal inhibitory concentration (IC50) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. The HCT116 human colon cancer cells and WISH human normal epithelial cells were treated with serial dilutions of GSE (23.43- 3000µg/ml). After 72hr, it was clear that GSE decreased the proliferation of HCT116 cells in concentration-dependent manner with an IC50 at 80µg/ml (Fig. 2). However GSE enhanced
the growth of the normal human epithelial cells WISH at low concentration and then GSE inhibited their proliferation at higher concentration with an IC50 = 2000µg/ml (Fig. 1).

Fig. 1: Determination of GSE cytotoxicity for normal WISH cell line by using SRB assay. Values were plotted by using GrabhPad PRISM program (GraphPAD, UK). IC50 (inhibitory concentration 50) = 2000µg.

Fig. 2: Determination of GSE cytotoxicity for canaer HCT116 cell line by using SRB assay. Values were plotted by using GrabhPad PRISM program (GraphPAD, UK). IC50 (inhibitory concentration 50) = 80µg.
GSE-Induced Growth Inhibition:

HCT116 cells were grown in 96-well assay plates and their proliferation was measured over 4 days, in four separate independent experimental trials, to determine the growth inhibitory effect of different doses of GSE (25, 50 and 100µg/ml). Growth inhibition was recorded every 24 hours until 96 hours. The results demonstrated that HCT116 cellular proliferation was inhibited by GSE in a dose- and time-dependent manner at 72-96 hrs in respect to untreated control cells (Fig. 3). While, results showed that GSE did not induce any detectable growth inhibitory effect on cultured HCT116 cells at 24-48 hrs.

Two tailed t-test, performed to validate the reduction of HCT116 proliferation at all GSE concentrations, revealed that all experimental concentrations resulted in statistically significant inhibition of proliferation. Whereas, GSE treatment at different doses (25, 50 and 100µg/ml) resulted in a significant growth inhibition of HCT116 cells ranging from 20-55% (P<0.05), 23-57.5% (P < 0.05-0.001) and 54-67% (P<0.001) after 96 hrs from treatment, respectively.

Expression of P53, BAX and BCL-2 mRNAs in HCT116 cultured cells:

Expression of P53, BCL-2 and BAX mRNAs in HCT116 cultured cells treated with different GSE doses (25, 50 and 100µg/ml) at different sampling time (24, 48, 72 and 96 hrs) is shown in Fig. 4. In GSE treated samples, expression of GAPDH gene remained unchanged with respect to control cells. Unexpectedly, the expression of P53 and BAX genes decreased in GSE treated HCT116 cells with a dose dependent manner at 24-48 hrs sampling time then their expression up-regulated again at 72-96 hrs at a dose dependent manner. While the expression of BCL-2 up-regulated at 24-48 hrs then down-regulated again at 72-96 hrs sampling time at a dose dependent manner with respect to control untreated cells.

GSE induced DNA fragmentation:

DNA fragmentation is a marker of late stage apoptosis. DNA fragmentation of HCT116 colon cancer cells was induced by GSE treatment in a dose- and time-dependent manner but it appears more effective at 72-96hrs (Fig. 5).

Discussion:

In the last decade, several studies have documented the anticancer and cancer chemopreventive efficacy of GSE against various cancers, including human colon tumors (Dinicola et al., 2010); these effects are usually attributed to the epigallocatechin and procyandins content of GSE (Agarwal et al., 2007; Inaba et al., 2008).
However, the major caveats had been the composition of various preparations of GSE being marketed under different names, and those being used under laboratory conditions. Furthermore, the lack of standardized preparations has also limited the validity and translational potential of the research findings obtained in the laboratory setting using different preparations/source of GSE (Kaur et al., 2008).

![Agarose gel (1.5%) separating RT-PCR of P53, BAX and BCL2 genes for Colon HCT116 cancer cells (b,c and respectively). In which lane 1 represents untreated cells, lanes (2-4) represent GSE treatment at 24h sampling time for 25, 50 and 100 µg/ml respectively, lanes (5-7) represent GSE treatment at 48h sampling time for 25, 50 and 100 µg/ml respectively, lanes (8-10) represent GSE treatment at 72h sampling time for 25, 50 and 100 µg/ml respectively, lanes (11-13) represent GSE treatment at 96h sampling time for 25, 50 and 100 µg/ml respectively, Fig. 4a: Representative 1.5% agarose gel separating RT-PCR of GAPDH for Colon HICT116 cancer cells (lanes 1-5).](image)

In the present study, we compared the cytotoxic effect of GSE procured from XIAMEN against human colon cancer HICT116 and normal epithelial WISH cell lines. It was clear that GSE enhanced the growth of the normal human epithelial cells WISH at low concentration and then it inhibited their proliferation at higher concentration with an IC50 =2000µg/ml. However, it was estimated that GSE decreased the proliferation of HICT116 cells in concentration-dependent manner with an IC50 at 80µg/ml. This indicates that GSE preferentially target cancer cells while sparing their normal counterparts. The exact reason for this is not clear but could be due to differential metabolism of bioactive compounds in normal and cancer cells that remains to be established (Lu et al., 2001; Jayaprakasha et al., 2010). These results were in agreement with Ye et al. (1999), they evaluated that grape seed proanthocyanidin extract enhanced the growth and viability of the normal human gastric mucosal cells and J774A.1 murine macrophage cells, while exhibited cytotoxicity towards some cancer cells (MCF-7 breast cancer, A-427 lung cancer and gastric adenocarcinoma cells) in a concentration- and time-dependent manner. Since we estimated that GSE exhibited no cytotoxicity toward the normal WISH cells, except at higher concentration, which is out of our concentration study range, therefore we proceed in the experimental study using HICT116 colon cancer cells only.
Fig. 5: Agarose gel (2%) separating extracted DNA (showing fragmentation or ladder) for Colon HCT116 cancer cells. In which lane M represents Low molecular weight DNA marker, lane 1 represents untreated cells, lanes (2-4) represent GSE treatment at 24h sampling time for 25, 50 and 100 ug/ml respectively, lanes (5-7) represent GSE treatment at 48h sampling time for 25, 50 and 100 ug/ml respectively, lanes (8-10) represent GSE treatment at 72h sampling time for 25, 50 and 100 ug/ml respectively, lanes (11-13) represent GSE treatment at 96h sampling time for 25, 50 and 100 ug/ml respectively.

We estimated the antiproliferative effect of GSE against HCT-116 cell line in a concentration- and time-dependent manner compared with the untreated cells. However, this growth inhibitory effect was declared at the higher sampling time but appeared less effective at 24-48hrs. Our results in agreement with Gabriela et al. (2008), in which the in vitro treatment with GSE proved to be efficient in all the experimental variants (24, 48, and 72hrs), but the most significant increase in number of apoptotic cells was registered after 72 hrs of treatment, when the maximum percentage of apoptosis (50, 40%) was obtained.

Several reports have suggested the proapoptotic BAX protein to act as a tumour suppressor in human malignancies (Nehls et al., 2007) playing a key role in mediating the apoptotic programme in response to genotoxic stress (Theodorakis et al., 2002). For this reason, we evaluated the BAX mRNA expression as a prognostic influence in HCT116 colon cancer cells treated by GSE with different doses at different sampling time. Additionally, we considered the interaction between proapoptotic BAX and its antiapoptotic counterpart BCL-2 as well as its proposed transcriptional upstream regulator p53 in these series of experiments. In addition, we evaluated the apoptotic effect of GSE by using DNA fragmentation assay.

In the present study, we demonstrated a reduction in BCL-2 mRNA expression and increase in BAX and p53 mRNAs expression that had been treated with GSE. It was clear that the effect of GSE in down-regulation of BCL-2 and up-regulation of BAX and P53 mRNAs expression appeared at 72-96hrs from treatment at different doses (25, 50 and 100µg/ml). Since p53 has also been shown to have a direct effect on apoptosis in vitro by regulating BCL-2 and BAX expression, our observations were consistent with the increased apoptotic changes in tumor cells. In which, DNA fragmentation, as an indication of late stage apoptosis, of HCT116 cells due to GSE treatment appeared to be dose- and time-dependent especially at 72-96hrs compared from control untreated cells.

Several studies have suggested that cancer chemotherapeutic drugs induce apoptosis of tumor cells, in part, by inducing the formation of reactive oxygen species (ROS) (Low et al., 2010). The p53 tumor suppressor gene can induce either apoptosis or senescence in response to cellular stresses. ROS accumulation and mitochondrial function can contribute to p53-dependent apoptosis and it has been shown in multiple studies that ROS inducers like bioactive agents collaborate with p53 to influence apoptosis (Zhou et al., 2008). Also, recent articles have pointed out the importance of the BAX, BCL-2 and ROS production in tumor cells (Chen and Pervaiz, 2007).
Hence, collectively, our results indicated that anticancer potency of GSE obtained from XIAMEN exhibits some differences from other previous reported studies (using different GSE cultivars), that cannot be explained only in terms of their relative epigallocatechin or procyanidin content, and other compounds seemingly can enhance (or even interfere with) the anticancer effects exerted by GSE (Dinicola et al., 2012). Indeed, it has been reported that GSE anticancer effects is linked to the pyrogallol-type structure and to the presence of a gallate ester moiety at”3”position of procyanidin B2. So far, differences in the relative concentration of compounds like these could be related to their respective differences in biological activities. Furthermore, GSE-related antineoplastic effects are highly specific for cancer type, as previously reported (Hsu et al., 2009). In addition, Dinicola et al., (2012) reported that HCT-8 colon cancer cells are differently sensitive to the anticancer effects triggered by GSE than Caco2 colon cancer cells.

In summary, the current study revealed that GSE works to suppress proliferation and induce apoptosis in human HCT116 colon cancer cells via p53 dependent mechanisms, but not in normal WISH epithelial cells, using different doses at different sampling time indicating that GSE preferentially target cancer cells. GSE exerts its effect in a dose- and time-dependent manner especially at 72-96hrs.

References


