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Original Research Communication

Amelioration of the Cytotoxic Effects of Chemotherapeutic Agents by Grape Seed Proanthocyanidin Extract

SHANTARAM S. JOSHI,[1] CHARLES A. KUSZYNSKI,[1] ERIC J. BENNER,[1] MANASHI BAGCHI,[2] and DEBASIS BAGCHI[2]

ABSTRACT

Anticancer chemotherapeutic agents are effective in inhibiting growth of cancer cells *in vitro* and *in vivo*, however, toxicity to normal cells is a major problem. In this study, we assessed the effect of a novel IH636 grape seed proanthocyanidin extract (GSPE) to ameliorate chemotherapy-induced toxic effects in cultured Chang epithelial cells, established from nonmalignant human tissue. These cells were treated *in vitro* with idarubicin (Ida) (30 n*M*) or 4-hydroxyperoxycyclophosphamide (4HC) (1 μg/ml) with or without GSPE (25 μg/ml). The cells were grown *in vitro* and the growth rate of the cells was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue] assay. Our results showed that GSPE decreased the growth inhibitory and cytotoxic effects of Ida as well as 4HC on Chang epithelial cells *in vitro*. Because these chemotherapeutic agents are known to induce apoptosis in the target cells, we analyzed the Chang epithelial cells for apoptotic cell population by flow cytometry. There was a significant decrease in the number of cells undergoing apoptosis following treatment with GSPE. We also found increased expression of the anti-apoptotic protein Bcl-2 in GSPE-treated cells using western blot techniques. Thus, these results indicate that GSPE can be a potential candidate to ameliorate the toxic effects associated with chemotherapeutic agents and one of the mechanisms of action of GSPE includes upregulation of Bcl-2 expression. Antiox. Redox Signal. 1, 563–570.

INTRODUCTION

PROANTHOCYANIDINS, naturally occurring compounds that are widely available in fruits, vegetables, nuts, seeds, flowers, and bark, represent a group of polyphenolic bioflavonoids diverse in chemical structure, pharmacology, and characteristics (Rice-Evans *et al.*, 1996; Rice-Evans and Packer, 1997). Proanthocyanidins have been reported to exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, antiallergic, and vasodilatory actions (Buening *et al.*, 1981; Afanas'ev *et al.*, 1989; Kolodziej *et al.*, 1995). Furthermore, proanthocyanidins have been reported to inhibit lipid peroxidation, platelet aggregation, capillary permeability, and fragility, and modulate the activity of enzyme systems including cyclooxygenase and lipooxygenase (Bors and Saran, 1987; Kolodziej *et al.*, 1995).

Chemotherapeutic agents are potent cytotoxic drugs used against various cancers. A combination of two or more compounds having specificity for different phases of cell cycle is commonly used to induce remission in cancer patients (Benner *et al.*, 1997; Rao *et al.*, 1997). Combination chemotherapy is often associated with treatment related toxicity to normal organs and tissue such as in the hematopoietic and gastrointestinal systems (Benner *et al.*,

[1]Departments of Cell Biology and Anatomy, Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198-6395.
[2]Creighton University School of Pharmacy and Allied Health Professions, Omaha, NE 68178.

1997; Rao *et al.*, 1997). These toxicities can be fatal if not properly managed. In patients who develop resistance to conventional doses of chemotherapy, high-dose therapy followed by stem cell transplantation is a standard method of treatment (Shea, 1999). In addition, chemotherapeutic agents either alone or in combination with other agents are used to eliminate or purge contaminated leukemic cells present in stem cell harvest for transplantation (Benner *et al.*, 1997; Rao *et al.*, 1997). During such purging, stem cells are often damaged. Oxidative damage represents a major mechanism of cytotoxicity induced chemotherapeutic agents (Benner *et al.*, 1997). Thus, cancer chemotherapy is invariably associated with significant damage to normal healthy organs and tissues (Benner *et al.*, 1997, Rao *et al.*, 1997). Therefore, development of new and effective strategies to diminish normal tissue toxicity is essential. In this context, we hypothesized that novel grape seed proanthocyanidin extract (GSPE) might be useful because it has been shown to serve as a free radical scavenger and antioxidant in both *in vitro* and *in vivo* models (Bagchi *et al.*, 1997, 1998a,b, 1999a,b). In a previous study, we demonstrated the cytotoxic effect of a novel IH636 GSPE toward selected human cancer cells, including MCF-7 breast cancer cells, A-427 lung cancer cells, and CRL-1739 human gastric adenocarcinoma cells. In contrast, the extract enhanced the growth and viability of a primary culture of normal human gastric mucosal cells and J774A.1 murine macrophage cells (Ye *et al.*, 1999). Furthermore, GSPE protected against smokeless tobacco-induced oxidative stress and apoptotic cell death in a primary culture of human oral keratinocytes (Bagchi *et al.*, 1999a), acute and chronic stress-induced oxidative gastrointestinal injury in rats (Bagchi *et al.*, 1999b), and acetaminophen overdose-induced lethality and hepatotoxicity (Ray *et al.*, 1999). In this study, we sought to investigate the chemopreventive effects of GSPE against idarubicin (Ida)- and 4-hydroxyperoxycyclophosphamide (4HC)-induced toxicity toward Chang epithelial cells, isolated and cultured from nonmalignant human tissue (Chang, 1954).

MATERIALS AND METHODS

Chemicals

A dried, powdered IH636 GSPE (ActiVin batch no. 609016) was obtained from InterHealth Nutraceuticals Incorporated (Concord, CA). GSPE is a standardized water-ethanol extract from red grape seeds. This extract contains antioxidants including catechins, which are oligomeric proanthocyanidins known to accumulate in the seeds (Kovac *et al.*, 1995). High-performance liquid chromatography (HPLC) studies in conjunction with gas chromatography-mass spectrometry (GC-MS) demonstrated that GSPE contains mostly dimeric, trimeric, and tetrameric proanthocyanidins and a small amount of flavonoids. The relative content of each of these compounds in GSPE are shown in Table 1. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue] was purchased from Sigma Chemical Co. (St. Louis, MO) (Catalog #M-2128). RPMI-1640 medium was obtained from GIBCO BRL (Gaithersburg, MD) and supplemented with 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, and antibiotics penicillin (100 U/ml) streptomycin (100 μg/ml). This medium is referred as RF10 medium. Idarubicin was a generous gift from Pharmacia (Upsala, Sweden). 4HC was a generous gift from Dr. M. Colvin of Duke Comprehensive Cancer Center (Durham, NC). Sodium dodecyl sulfate (SDS) was purchased from Boehringer Manheim Biochemicals (Indianapolis, IN) *N,N*-dimethyl formamide (DMF) was purchased from Fisher (Fair Lawn, NJ). Unless otherwise stated, all other chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or the highest grade available.

TABLE 1. COMPOSITION OF GRAPE SEED PROANTHOCYANIDIN EXTRACT

Constituents	*Percentage*
Oligomeric proanthocyanidins	
Dimeric (chain of 2)	54%
Trimer (chain of 3)	13%
Tetramer (chain of 4)	7%
Monomers and other flavonoids	6%

In vitro *cell culture system*

Chang cells (American Type Culture Collection, Rockville, MD; CCL-13) were established from nonmalignant human epithelial tissue by Dr. R.S. Chang (Department of Microbiology, Harvard School of Public Health, Boston, MA) in 1954. The Chang cells used in this study were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 m*M* L-glutamine, and the antibiotics streptomycin and penicillin. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were incubated *in vitro* under regular culture conditions at 37°C for varying periods of time with or without chemotherapeutic agents and various concentrations of GSPE (as indicated in the respective figure legends). The viability of cells was determined using MTT assay as described below. For the growth stimulatory effects of GSPE, the Chang cells were cultured in a 75-cm^2 flask with or without various concentrations of GSPE (as shown in figure legends) for 48 hr. At the end of the incubation period, cells were counted using an electronic counter and growth rate was plotted.

MTT assay

The cleavage of the tetrazolium salt MTT into a blue-colored formazan by the mitochondrial enzyme succinate dehydrogenase was used for determining cell survival and proliferation (Mosmann, 1983). Growth of Chang cells in the presence of chemotherapeutic agents and GSPE was determined by MTT assay. Cells were seeded at 2×10^3 cells/well in RF10 media in triplicate wells with or without the anticancer drugs and GSPE on a 96-well flat-bottomed microculture plate (Becton Dickinson Labware, San Jose, CA). After incubating the plates at 37°C in 5% CO_2 for 1, 3, or 5 days, MTT assays were performed. MTT was prepared at a concentration of 5 mg/ml in sterile PBS. Extraction/lysis buffer was prepared by dissolving 20% wt/vol of SDS at 37°C in a solution of 50% vol/vol each of DMF and demineralized water. A 25-μl aliquot of the stock solution of MTT was added to each well. After 2 hr of incubation at 37°C, 100 μl of extraction/lysis buffer was added to each well and mixed thoroughly to absorb formazan crystals. Optical densities were measured at 570 nm using a 96-well Multiscanner (Dynatech Instruments, Inc, Torrance, CA). A well containing RF10 medium, MTT, and extraction buffer in the absence of Chang epithelial cell extract was used as the blank (Mosmann 1983; Rao *et al.*, 1997).

Flow cytometric analysis of apoptosis

Chang epithelial cells either treated or not with chemotherapeutic agents were analyzed for apoptotic cell population by flow cytometry as previously described (Telford *et al.*, 1991; Bagchi *et al.*, 1999a). Briefly, the cells were harvested by trypsinization, counted, and fixed in 70% ethanol. The cells were resuspended in 2 ml of Telford reagent containing EDTA (67.24 μg), RNase (53.6 μg), propidium iodide (100 μg), Triton X-100 (2.0 μl), and phosphate-buffered saline (PBS) (to a total volume of 2 ml) (Telford *et al.*, 1991). Cells were incubated at 4°C for 24 hr and then analyzed using a FACSTAR Plus Flow Cytometer and Cell Quest Acquisition Software (Becton Dickinson, San Jose, CA). The percentage of cells within the G_0/G_1S and G/M phases of the cell cycle was determined by analysis of list mode data files with MODFIT LT software program (Variety Software House, Topshman, MO).

Western blotting for Bcl-2 protein expression

Cellular extracts were prepared by treating the cells with 0.1% *n*-octylglucoside in PBS (1×10^7 cells/ml) for 30 min at room temperature as described earlier by Hao *et al.* (1993). Following the incubation, the mixture was centrifuged at $200 \times g$ for 7 min. The supernatant containing cellular extract was then electrophoresed using 7% polyacrylamide gel, and the proteins were transferred to nylon membranes (Bio Rad, CA) using wet electroblotting techniques. The membrane containing the cellular extract protein was reacted with goat polyclonal IgG anti-Bcl-2 antibody as primary antibody, purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA), and peroxidase-conjugated anti-goat monoclonal antibody, purchased from KPL, Inc. (Rockville, MD) was used as a secondary antibody. Reac-

tive bands were identified using diaminobenzidine (DAB) as a substrate for formation of color reaction. The density of each band was then determined using Image Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) software and compared to the control value.

Statistical analysis

Statistical significance of the difference between the results was analyzed with the Students' *t*-test. Differences were considered significant when the p value was less than 0.05.

RESULTS

Amelioration of chemotherapy-induced toxicity

Figure 1 demonstrates the ameliorating effects of GSPE on idarubicin (Ida)-induced growth inhibition of Chang epithelial cells *in vitro*. When the Chang epithelial cells were treated with 30 n*M* concentration of Ida, there was a significant ($p < 0.05$) decrease in the growth of Chang *in vitro* as measured by the MTT assay. Ida-induced growth inhibition was not observed following incubation in the presence of GSPE at a concentration of 25 μg/ml. In this experiment, the *in vitro* growth analyses was followed by 5 days in culture. Figure 1 also shows similar protective effects of GSPE on 4HC-induced growth inhibition of Chang cells. In this experiment, 1 μg/ml concentration of 4HC significantly inhibited the growth of Chang epithelial cells. Cotreatment of GSPE at a concentration of 25 μg/ml together with the chemotherapeutic agent 4HC diminished the toxicity significantly ($p < 0.05$). To determine the effects of GSPE on the rate of growth of Chang cells, these cells were grown in the presence of various concentrations of GSPE. The growth rate of such GSPE-treated and -untreated cells was measured using an electronic cell counter (Coulter Electronics, Hileah, FL). Figure 2 shows the growth stimulatory effects of GSPE on the Chang cells. The results showed that 25 μg/ml of GSPE alone significantly stimulated ($p < 0.05$) the growth of Chang epithelial cells in regular culture.

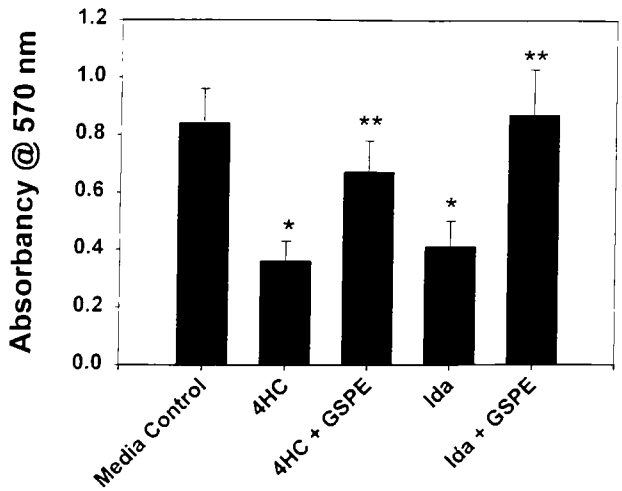

FIG. 1. Effect of the chemotherapeutic agents Ida (30 n*M*) or 4HC (1 μg/ml) with or without GSPE (25 μg/ml) on *in vitro* growth of Chang epithelial cells. Cells were incubated at 37°C with and without GSPE for 5 days in culture medium. The growth rate of cells was determined using the MTT assay. Values represent the mean of three separate experiments. *$p < 0.05$, significant decrease in growth compared to control value; **$p < 0.05$, significant increase in cell growth compared to Ida or 4HC treatment alone.

Effects of GSPE on apoptosis induced by chemotherapeutic agents

The chemotherapeutic agents used in this study are known to induce apoptosis, hence we analyzed for the number of Chang epithelial cells undergoing apoptosis in response to treatment with 4HC or Ida. Figure 3 demonstrates

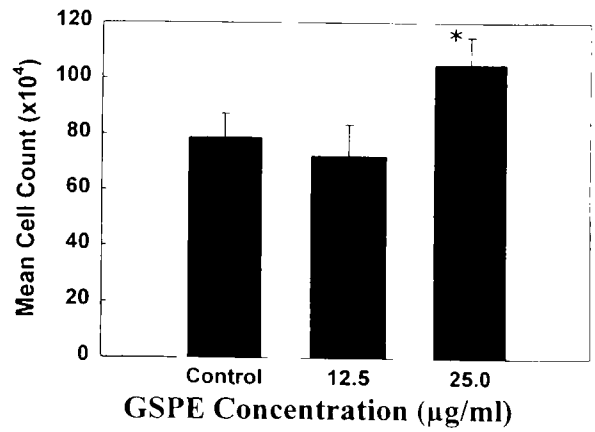

FIG. 2. Effects of GSPE on the *in vitro* growth of Chang epithelial cells. Cells were grown with different concentrations of GSPE and growth rate was determined by counting the cells 48 hr after *in vitro* culture. Values represents the mean of three separate experiments. *$p < 0.05$, significantly different from control.

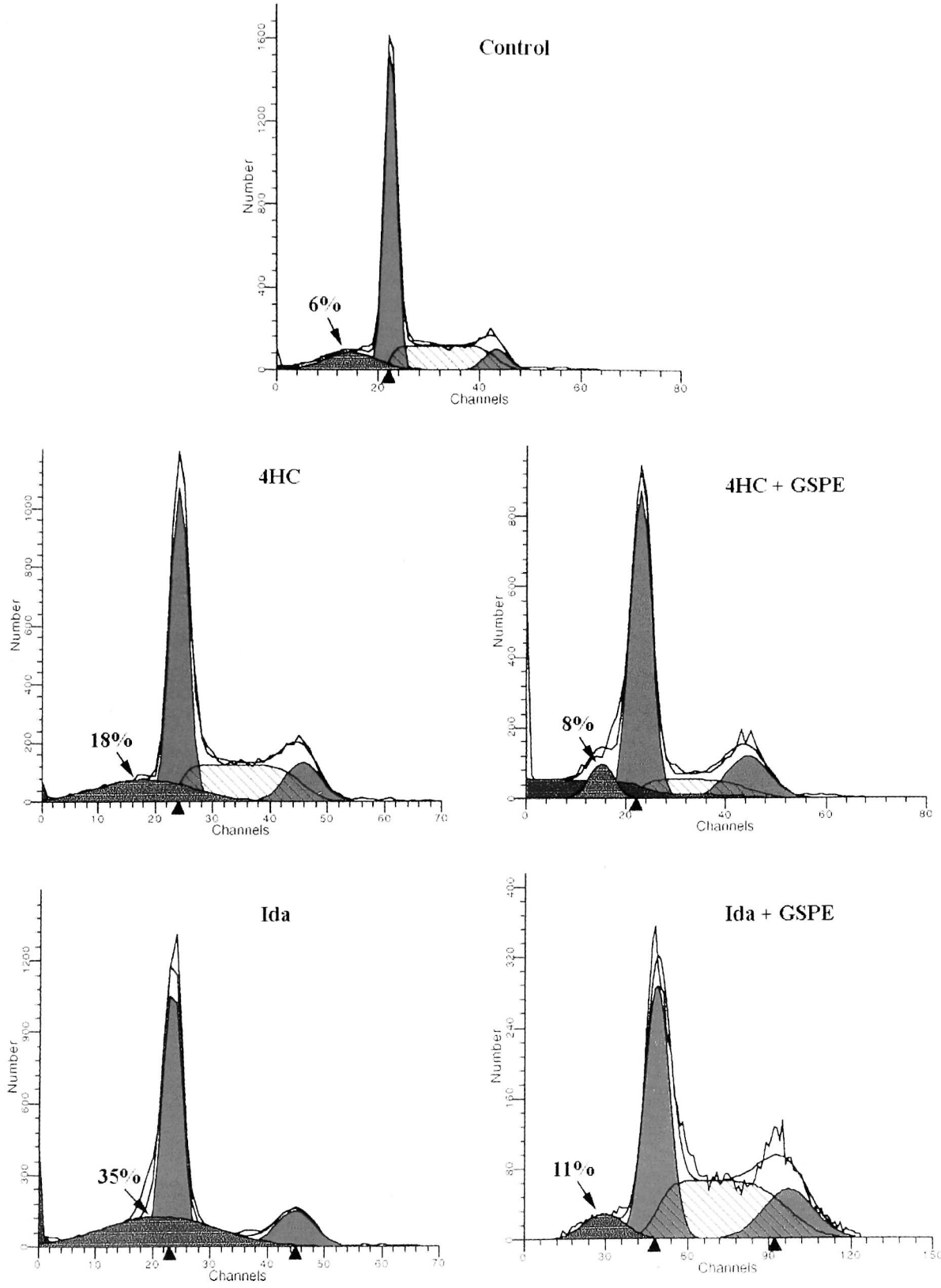

FIG. 3. Flow cytometric analysis of Chang epithelial cells for the detection of apoptosis. The cells were treated with 4HC (1 μg/ml) or Idarubicin (Ida) (30 nM) with or without GSPE (25 μg/ml) for 48 hr, and control and treated cells were analyzed for the percentage of cells undergoing apoptosis. **A**. Control untreated Chang cells. **B**. Chang cells treated with 4 HC (18% apoptotic cells shown by arrow). **C**. Chang cells co-treated with 4HC and GSPE (8% apoptotic cells shown by arrow). **D**. Chang cells treated with Ida (35% apoptotic cells shown by arrow) **E**. Chang cells co-treated with Ida and GSPE (11% apoptotic cells shown by arrow).

the flow cytometric analysis for apoptotic cell population. There were 18 ± 3% of cells undergoing apoptosis in Chang epithelial cells treated with 4HC for 48 hr. In control untreated Chang epithelial cells, 6 ± 1.5% of apoptotic cells were detected at that time point. When GSPE was added to the culture at a concentration of 25 μg/ml, the number of cells undergoing apoptosis was reduced to 8 ± 2% in 4HC-treated cells. Whereas 35 ± 4% of the Chang epithelial cells were apoptotic following treatment with Ida alone for 48 hr, when GSPE (25 μg/ml) was added in conjunction with Ida the number of cells undergoing apoptosis was reduced to 11 ± 3%.

Expression of Bcl-2

Because GSPE decreased both Ida as well as 4HC induced apoptosis, we analyzed these cells for the expression of the anti-apoptotic protein Bcl-2 using Western blot technique. Figure 4A shows Bcl-2 expression in Chang epithelial cells treated with Ida and/or GSPE. Ida treatment for 48 hr decreased Bcl-2 expression in these cells. The treatment of cells with GSPE prevented such Ida-induced downregulation of Bcl-2 expression (Fig. 4A). This protective effect of GSPE on Ida-treated Chang epithelial cells was confirmed by densitometric analysis using Image Pro analytical program (Fig. 4B).

DISCUSSION

The major objective of this study was to assess the protective role of GSPE against Ida or 4HC-induced cytotoxicity in cultured Chang epithelial cells, isolated and cultured from nonmalignant human tissue. The biological, pharmacological, and medicinal properties of bioflavonoids have been extensively reviewed (Shahidi and Wanasundara, 1992; Rice-Evans and Packer, 1997). Increasing interest in proanthocyanidins is based on a variety of pharmacological, medicinal, and therapeutic potential, including inhibition of DNA topoisomerase II, protein kinase C, angiotensin-converting enzyme, and hyaluronidase enzyme activities (Hanefeld and Herrmann, 1976; Masquelier *et al.*, 1979; Chen *et al.*, 1996; Rice-Evans and Packer, 1997). Proanthocyanidins also possess potent antibacterial, antiviral, and anti-HIV

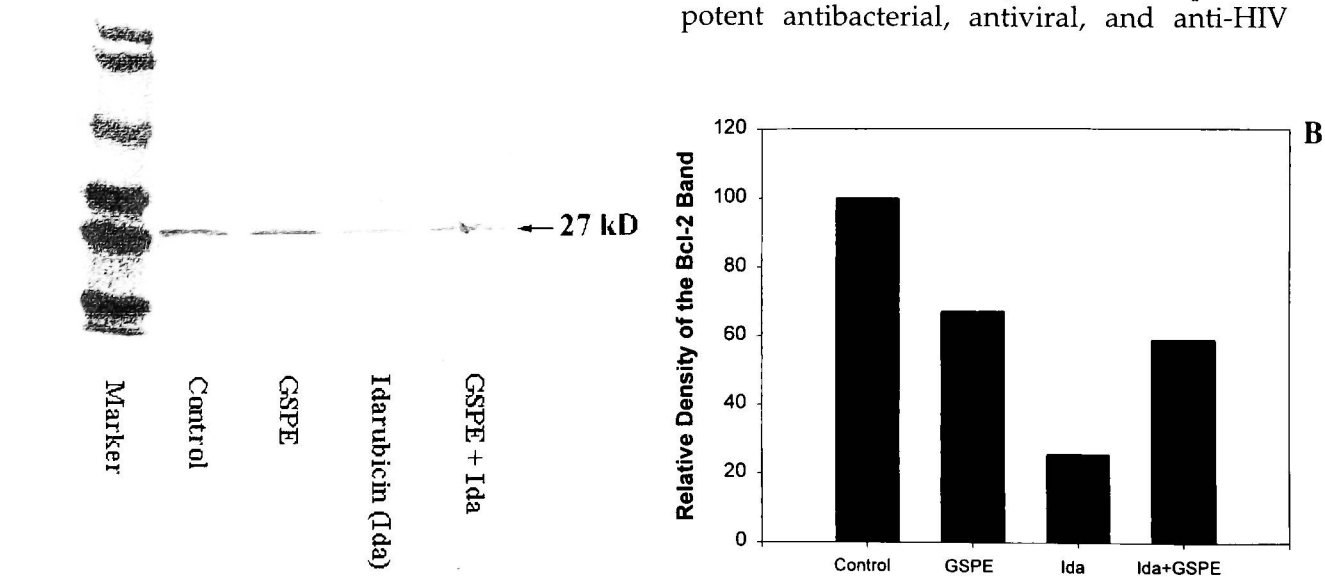

FIG. 4. Bcl-2 expression. A. Western blot analysis of Chang epithelial cells treated with Ida with or without GSPE for 48 hr. The cell extracts were prepared and separated on polyacrylamide gel electrophoresis, and proteins were transferred onto nylon membrane and reacted with antibodies to Bcl-2. The reacted antibodies were identified using enzyme-conjugated second antibodies and substrate. **B.** Densitometric analysis of Bcl-2 expression in cells treated with Ida or Ida plus GSPE as shown in **A.** The densitometric analysis was performed using Image Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) analytical program.

activities (Kolodziej *et al.*, 1995). It has been demonstrated that proanthocyanidins exhibit antihypertensive effects (Rice-Evans and Packer, 1997), anti-peptic activity, monocyte-stimulating ability, and anti-hepatotoxic activity (Kolodziej *et al.*, 1995). The chemical properties of bioflavonoids, in terms of the availability of the phenolic hydrogens as hydrogen-donating radical scavengers and singlet oxygen quenchers, predicts their antioxidant activity (Shahidi and Wanasundara 1992; Chen *et al.*, 1996; Rice-Evans *et al.*, 1996; Rice-Evans and Packer, 1997). Proanthocyanidins have also been demonstrated as potent inhibitors of the enzymes phospholipase A_2, cyclooxygenase and lipooxygenase (Bors and Saran, 1987; Kolodziej *et al.*, 1995; Salah *et al.*, 1995; Rice-Evans *et al.*, 1996). Thus, proanthocyanidins or polyphenolic bioflavonoids may act as antioxidants, and/or by other mechanisms, may contribute to chemoprotective and health benefits.

In our previous studies, we have demonstrated concentration- or dose-dependent free radical scavenging abilities of GSPE, in both *in vitro* and *in vivo* models (Bagchi *et al.*, 1997, 1998a,b, 1999a,b). These studies demonstrated that GSPE is bioavailable to the target tissues, including liver, brain, and peritoneal macrophages, and provides significantly greater protection against biochemically generated free radicals and free radical-induced lipid peroxidation and DNA damage than vitamin C, vitamin E, a combination of vitamins C plus E, and β-carotene (Bagchi *et al.*, 1997, 1998a,b, 1999a). GSPE also protected against acetaminophen overdose-induced liver injury (Ray *et al.*, 1999), tobacco-induced oxidative stress and apoptotic cell death in human oral keratinocytes (Bagchi *et al.*, 1999a), as well as against acute and chronic stress-induced gastrointestinal mucosal injury in rats (Bagchi *et al.*, 1999b). The present study demonstrated that GSPE may significantly decrease the growth inhibitory effects of Ida and 4HC on Chang epithelial cells *in vitro*. The number of cells undergoing apoptosis in Chang cells treated with chemotherapeutic drugs was significantly reduced by GSPE. Our results show that the GSPE diminishes cytotoxicity induced by chemotherapeutic drugs such as Ida and 4HC on the Chang epithelial cells. One of the possible mechanisms of action of GSPE includes preventing decrease of Bcl-2 expression induced by chemotherapeutic agents. In summary, the results of these experiments suggest that GSPE can significantly protect against Ida as well as 4HC-induced cytotoxicity toward cultured Chang epithelial cells, and may serve as a novel co-therapeutic agent in conjunction with Ida and 4HC. *In vivo* studies using tumor chemotherapy models are necessary to strengthen current findings.

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ABBREVIATIONS

DAB, diaminobenzidine; DMF, *N,N*-dimethylformamide; FBS, fetal bovine serum; GC-MS, gas chromatography-mass spectrometry; GSPE, grape seed proanthocyanidin extract; 4HC, 4-hydroxyperoxycyclophosphamide; HPLC, high-performance liquid chromatography; Ida, idarubicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline (pH 7.4); RF10, RPMI-1640 medium supplemented with 10% fetal bovine serum; SDS, sodium dodecyl sulfate.

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Address reprint requests to:
Dr. Shantaram S. Joshi
Department of Cell Biology and Anatomy
University of Nebraska Medical Center
986395 Nebraska Medical Center
Omaha, Nebraska 68198-6395

E-mail: ssjoshi@unmc.edu

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