Cancer Chemopreventive Properties of Processed Ginseng

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ABSTRACT

Ginseng is one of the most widely used medicinal plants, particularly in East Asian countries. Certain fractions or purified ingredients of ginseng have been shown to exert inhibitory effects on growth of cancer cells in culture or on tumorigenesis in experimental animals. Moreover, a recent epidemiologic study reveals that ginseng intake is associated with a reduced risk for environmentally related cancers such as esophageal, gastric, colorectal, and pulmonary tumors. Heat treatment of *Panax ginseng* C. A. Meyer at the temperature higher than that applied to the conventional preparation of red ginseng yielded a mixture of saponins with potent antioxidative properties. Thus, the methanol extract of heat-processed ginseng (designated as 'NGMe') attenuated lipid peroxidation in rat brain homogenates induced by ferric ion or ferric ion plus ascorbic acid. Furthermore, the extract protected against strand scission in ϕ X174 supercoiled DNA induced by UV photolysis of H_2O_2 and was also capable of scavenging superoxide generated *in vitro* by xanthine/xanthine oxidase or in differentiated human promyelocytic leukemia (HL-60) cells by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). Since tumor promotion is closely linked to oxidative stress, we have determined possible anti-tumor promotional effects of NGMe on two-stage mouse skin tumorigenesis. Topical application of NGMe onto shaven backs of female ICR mice 10 min prior to TPA significantly ameliorated skin papillomagenesis initiated by 7,12-dimethylbenz[a]anthracene (DMBA). Likewise, TPA-induced epidermal ornithine decarboxylase activity and elevation of tumor necrosis factor-α were suppressed significantly by NGMe pretreatment. NGMe topically applied onto surface of hamster buccal pouch 10 min before each topical application of DMBA inhibited oral carcinogenesis by 76% in terms of multiplicity. Taken together, these results suggest that processed *Panax ginseng* C. A. Meyer has potential cancer chemopreventive activities.

Introduction

Panax ginseng C. A. Meyer is one of the most widely used medicinal plants, particularly in East Asian countries. It has a wide range of pharmacological and physiological actions. Certain fractions or purified individual ingredients of ginseng have been shown to exert cytotoxic or cytostatic activi-

ties against tumor cells in culture (Matsunaga *et al.*, 1990; Kubo *et al.*, 1992; Baek *et al.*, 1996; Wakabayashi *et al.*, 1998). The growth inhibitory or anti-proliferative effects of certain ginsenosides appear to be associated with their capability to induce apoptosis (Park and Lee, 1997; Wakayabashi *et al.*, 1998). In addition, the antimetastatic potential of ginseng saponins has been evaluated in experimental animal tumor models (Sato *et al.*, 1994; Mochizuki *et al.*, 1995; Iishi *et al.*, 1997; Wakabayashi *et al.*, 1997). Ginsenoside Rb_2 from *Panax ginseng* has been shown to inhibit angiogenesis as well as metastasis produced by B16-BL6 melanoma cells in syngenic mice (Sato *et al.*, 1994). The methanolic extract of red ginseng enhanced the uptake of mitomycin C by tumor cells and enhanced its cytotoxic effect (Kubo *et al.*, 1992). Likewise, panaxytriol potentiated the mitomycin-induced cytotoxicity in cultured cancer cells (Matsunaga *et al.*, 1994). The root extracts of *Panax ginseng* also exhibit protective effects against mutation in Chinese hamster V79 cells and transformation of NIH3T3 cells induced by some chemical carcinogens (Rhee *et al.*, 1991). Ginsenoside Rh_2 present in red ginseng has been reported to suppress the formation of sister chromatid exchanges in human blood lymphocytes (Zhu *et al.*, 1995). Moreover, a recent epidemiologic study reveals that ginseng intake is associated with a reduced risk for environmentally related cancers such as esophageal, gastric, colorectal, and pulmonary tumors (Yun and Choi, 1990 & 1995; Yun, 1996). *Panax ginseng* extracts have been reported to induce hepatic epoxide hydrolase and glutathione S-transferase (Lee *et al.*, 1987) which are involved in detoxification of ultimate electrophilic metabolites of such chemical carcinogens as benzo[a]pyrene and aflatoxin B_1. However, only limited data are available in the literature with regard to chemopreventive activities of ginseng in experimental carcinogenesis (Yun *et al.*, 1983, 1987, 1993; Yun, 1998; Xiaoguang *et al.*, 1998). Heat-treatment of ginseng at temperature and under pressure higher those applied to the conventional preparation of red ginseng produces a new processed ginseng with fortified antioxidative activity. As part of a program aimed at developing new types of naturally occurring chemopreventive agents, we initially evaluated antioxidative activity of the methanol extract of the new processed ginseng (abbreviated as NGMe) and also examined its effects on chemically-induced carcinogenesis in experimental animals.

Materials and Methods

Materials

12-O-Tetradecanoylphorbol-13-acetate (TPA) was a product of Alex Biochemicals (San Diego, CA). Thiobarbituric acid (TBA), nitroblue tetrazolium (NBT), cytochrome c (Cyt. c), ϕ X174 RF1 supercoiled DNA, sodium dodecylsulfate (SDS), proteinase K, and 7,12-dimethylbenz[a]anthracene (DMBA) were purchased from Sigma Chemical Co. (St. Louis, MO). DL-[1-^{14}C]Ornithine was obtained from Dupont/NEN Research Products (Boston, MA). RPMI 1640 and fetal bovine serum

obtained from Dupont/NEN Research Products (Boston, MA). RPMI 1640 and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY). Other chemicals and solvents used were of highest analytical grade. NGMe prepared by methanol extraction of the new processed ginseng was supplied by Prof. Jeong Hill Park of the Seoul National University.

Lipid peroxidation in rat brain homogenates

Brain homogenates from young adult male Sprague-Dawley rats were prepared in 0.15M KCl (10%, w/v) and centrifuged at 800 g for 10 min, and the supernatant was used for *in vitro* lipid peroxidation assays. The incubation mixture in a final vol. of 1 ml contained brain homogenate (0.5 ml), 0.15M KCl and varying amounts of NGMe in deionized water. Lipid peroxidation was initiated by addition of ferric chloride (100 μM) alone or in combination with ascorbic acid (100 μM). After incubation for 20 min at 37℃, the reaction was terminated by addition of 0.5 ml cold trichloroacetic acid (5.5%) and the equal vol. of TBA (2% in 0.05M NaOH) followed by heating at 100℃ for 10 min. The mixtures were then centrifuged at 1,000 g for 10 min and the absorbance of the supernatant was measured at 532 nm.

Superoxide anion generation by xanthine-xanthine oxidase

Superoxide anion was generated by xanthine-xanthine oxidase and detected by the NBT reduction method (Aruoma *et al.*, 1984). The standard reaction mixture in a final vol. of 1 ml contained potassium phosphate buffer (0.1M, pH 7.8), xanthine (1 mM) and NBT (0.5 mM). The reaction was initiated by addition of xanthine oxidase (0.03 unit). After incubation at room temperature for 10 min, the absorbance at 560 nm was read.

Analysis of hydroxyl radical-induced DNA strand scission

DNA cleavage by reactive oxygen species generated by hydrogen peroxide and UV was measured by agarose gel electrophoresis. In brief, the reaction mixture (30μl) contained 10 mM Tris-1 mM EDTA buffer, pH 8.0, ϕ X 174 RF1 DNA (0.3 g), and H_2O_2 (0.03M). Hydroxyl radical was generated by UV photolysis as described above. After incubation at room temperature for 30 min, the reaction was terminated by addition of loading buffer (0.25% bromophenol blue and 40% sucrose) and mixtures were analyzed by 0.8% submarine agarose gel electrophoresis (70V, 1h). The gels were stained with ethidium bromide, destained in water, viewed and photographed under a transilluminator.

Epidermal ornithine decarboxylase (ODC) assay

The ODC activity in epidermal soluble fraction was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]Ornithine (43.8 mCi/mmol) as described previously. The protein concentration was

determined by the method of Bradford using bovine serum albumin as a standard.

ELISA assay for TNF- α production in mouse skin

The assay for determining the amounts of TNF- αwas performed by using a kit from Amersham Life Science (Buckinghamshire, UK) according to the manufacturer's instruction.

Induction of mouse skin carcinogenesis

Groups of 25-30 female ICR mice obtained from Korea Laboratory Animals Co. (Taejeon, Korea) were treated on their shaven backs with a single topical application of DMBA (0.2 μmol) in 0.2 ml acetone or the solvent alone. One week after the initiation, 15 nmol of TPA was topically applied twice weekly until termination of the experiment. NGMe dissolved in DMSO-acetone was topically applied 30 min before each TPA treatment. The control animals were pretreated with the solvent alone. Starting one week following the promoter treatment, tumors of at least 1 mm in diameter were counted every week.

Measurement of phorbol ester-induced superoxide anion generation in differentiated HL-60 cells

Inhibitory effects of NGMe on TPA-induced superoxide generation were determined in differentiated human promyelocytic leukemia (HL-60) cells according to previously reported procedures with minor modification. Briefly, cells were suspended at a density of 5 x 10^5 cells/ml in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 1.3% DMSO and incubated for 6 days at 37℃ in a humidified atmosphere of 5% CO_2-95% air. Differentiated cells were harvested by centrifugation, washed with and resuspended in PBS (1 x 10^6 cells/ml). Cells were preincubated for 10 min with and without NGMe, followed by addition of TPA (8 μM) and cyt. c (60 μM). After 30 min incubation at 37℃, superoxide generation was determined by measuring the absorbance of reduced cyt. c at 550 nm.

Results and Discussion

The anitoxidative and free radical scavenging effects of ginseng and some of its selected ingredients have been extensively investigated and well documented. Thus, ginseng extracts exhibited protective effects against peroxidation of unsaturated fatty acid caused by iron and hydrogen peroxide (Zhang *et al.*, 1996). Ginsenosides also protect pulmonary vascular endothelium from free radical-induced injury (Kim *et al.*, 1992). Panaxadiol fractions were found to significantly induce the expression of Cu, Zn-superoxide dismutase at the transcriptional level (Kim *et al.*, 1996), which also account for the antioxidative properties of ginseng observed by other investigators. Certain saponins derived from red ginseng, such as ginsenosides Rb_1, Rb_2, Rc, Rd, and Rg_1, attenuated carbon tetra-

chloride-induced lipid peroxidation in rat liver microsomes (Kim *et al.*, 1997) as well as hepatic lesions *in vivo* (Jeong *et al.*, 1997). Since carbon tetrachloride is known to be metablically activated by cytochrome P-450 2E1, suppression of this isoform by the aforementioned red ginseng saponins has been proposed as a plausible mechanism underlying their antioxidative and hepatoprotective effects against carbon tetrachloride (Kim *et al.*, 1997). In the present study, the antioxidative activity of NGMe was initially evaluated by determining its effect on lipid peroxidation in rat brain homogenates induced by ferric ion alone or feriic ion plus ascrobic acid. As summarized in Table 1, NGMe significantly attenuated the formation of thiobarbituric acid reactive substances (TBARS).

Table 1. Effect of NGMe on lipid peroxidation in rat brain homogenate

	% Inhibition	
NGMe (mg/ml)	Fe (III)	Fe (III) + Ascorbic acid
0.25	2.7 ± 1.5	56.0 ± 16.7
0.5	30.0 ± 13.7	89.8 ± 2.6
1.0	93.5 ± 0.95	97.2 ± 0.5
2.0	97.7 ± 0.6	99.8 ± 0.2

Values are mean ± S.D. Data are corrected for the spontaneous peroxidation without inducing agents.

Almost complete inhibition of lipid peroxidation was observed at 2 mg/ml of NGMe. Xanthine-xanthine oxidase generates superoxide anion which reduces NBT to yield blue formazan. NGMe inhibited dose-dependently the NBT reduction induced by xanthine-xanthine oxidase. Since NGMe did not influenced the activity of xanthine oxidase (data not shown), its inhibitory effect on formazan formation is likely to be attributable to scavenging of superoxide anions. In another experiment, the superoxide levels formed in differentiated HL-60 cells by phorbol ester was reduced by NGMe in a dose-related manner (Figure 1).

Superoxide anions are converted by superoxide desmutase to hydrogen peroxide that can subsequntly generates extremely reactive hydroxyl radicals. Formation of hydroxyl radicals from H_2O_2 can be stimulated by certain transition metal ions such as iron and copper or by UV photolysis. Hydroxyl radicals can attack DNA to cause strand scission. Thus, incubation of ϕX174 supercoiled DNA with both H_2O_2 and UV resulted in complete conversion of supercoiled DNA (Form III) to open circular (Form II) and subsequently linear (Form III) forms (Figure 2). UV illumination alone was not effective in causing DNA strands cleavage whereas treatment of ϕX 174 DNA with H_2O_2 led to partial conversion to Form I DNA, which appeared to be catalyzed by trace amounts of transition metal ions present in the solution. Addition of NGMe to the reaction mixture substantially diminished the DNA strand scission induced by both H_2O_2 and UV. There was almost complete protection by NGMe at a dose of 1 mg.

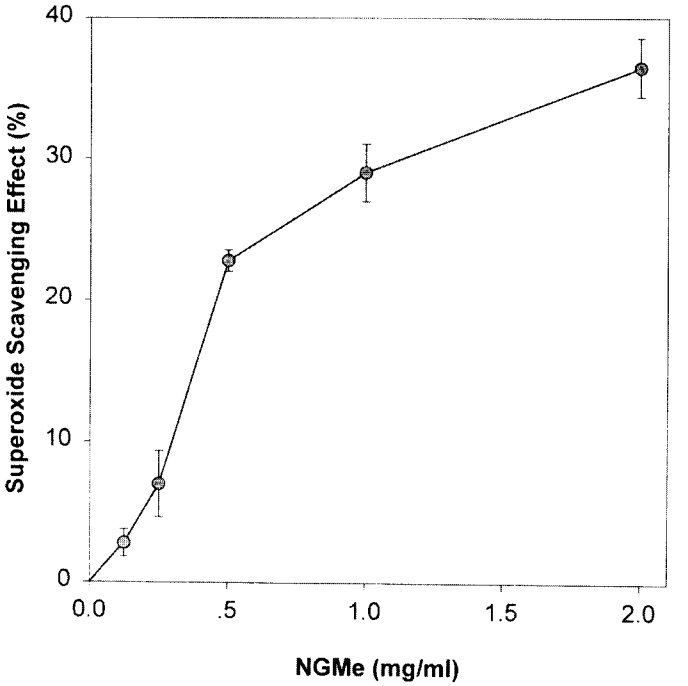
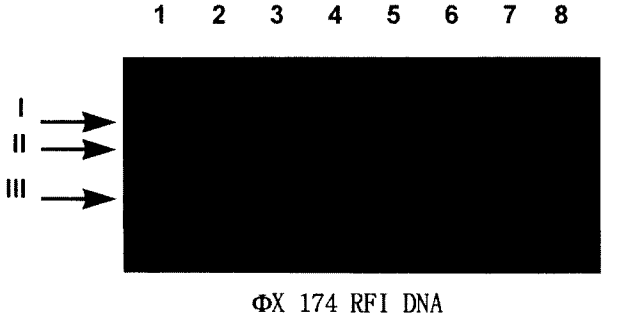


Fig 1. Inhibitory effect of the methanolic extract of the processed ginseng on TPA-stimulated production of superoxide radical in differentialted HL-60 cells. Experimental conditions are described in materials and Methods



I : Open circular DNA
II : Linear DNA
III : SupercoiledDNA

1 : No treatment
2 : H_2O_2 alone
3 : UV alone
4 : H_2O_2 + UV
5 : 4+NGMe 0.008mg
6 : 4+NGMe 0.04mg
7 : 4+NGMe 0.2mg
8 : 4+NGMe 1mg

Fig 2. Dose-related inhibition by NgMe of hydroxyl radical-mediated strand scission in ϕ X174 RF1 supercoiled DNA. Hydroxyl radical was generated by UV photolysis of hydrogen peroxide as described under Materials and Methods.

Reactive oxygen species (ROS) have been considered to play a role in multistage carcinogenesis (Klaunig *et al.*, 1998). There has been accumulated evidence for the involvement of ROS to pomote as well as initiate experimental carcinogenesis. Since NGMe has ability to scavenge ROS including superoxide and hydroxyl radicals, it is conceivable that this material possesses anti-tumor promotional potential. In our preliminary experiment, NGMe attenuated the tumor promoter-stimulated production of superoxide in differentiated HL-60 cells. Topical application of NGMe prior to each topical dose of TPA onto dorsal skins of female ICR mice gave rise to dramatic amelioration of skin tumor promotion (Figure 3). It is of interest to note that pretreatment with the higher dose (abbreviated as 0.5% Gin in the figure) of NGMe almost completely suppressed the mouse skin tumorigenesis in terms of both incidence and multiplicity. ODC is a rate-limiting enzyme in biosynthesis of polyamines that play pivotal roles in cell proliferation. Elevation of ODC activity is closely associated with tumor promotion. NGMe mitigated the TPA-induced elevation of epidermal ODC activity (Figure 4), which may provide mechanistic basis underlying its chemopreventive properties against mouse skin carcinogenesis. Since inflammation is recognized as an early event in multi-stage carcinogenesis, particularly in the tumor promotion step, anti-inflammatory effects of NGMe may also account for its inhibition of skin carcinogenesis. In support of this assumption, pretreatment of NGMe significantly lowered the TPA-stimulated production of TNF- α (Figure 5), one of the major cytokines mediating the tissue inflammation. Further studies will be necessary to elucidate the structural identity of the active chemopreventive components of NGMe and to clarify their molecular mechanism of action.

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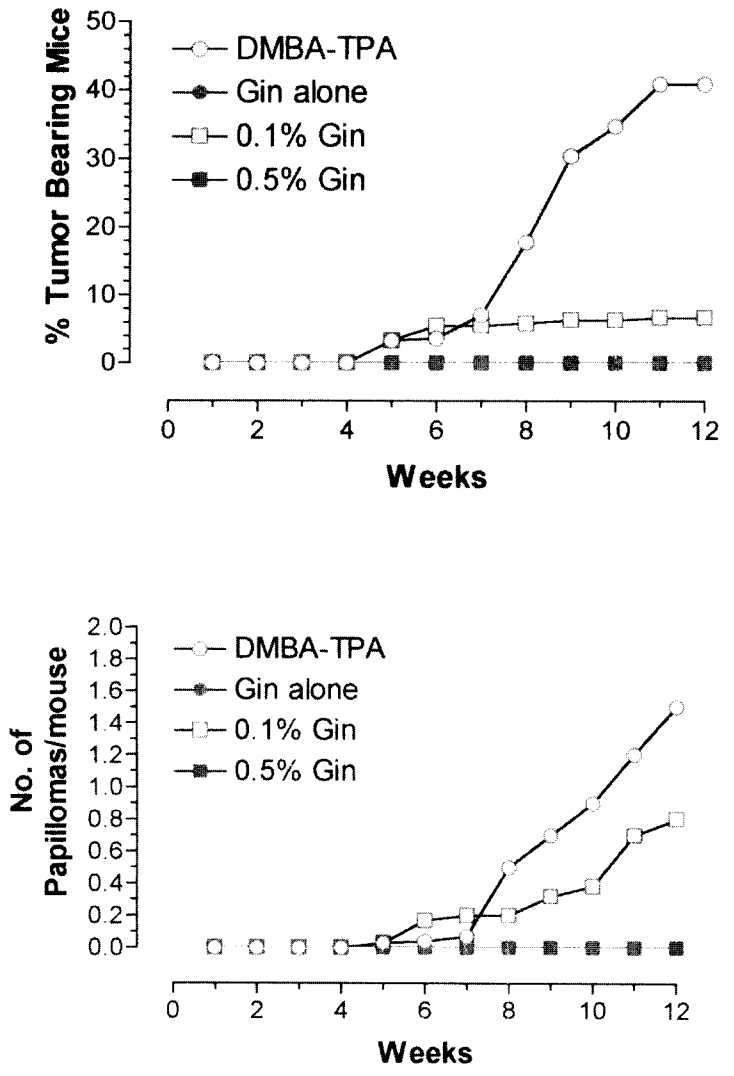

Fig 3. Inhibition of mouse skin tumor promotion by the methanolic extract of processed ginseng. Female ICR mice were treated topically with an indicated amount of the methanolic extract of processed ginseng (abbreviated as "Gin") 30 min prior to each topical dose of 15 nmol TPA after initiation with DMBA.

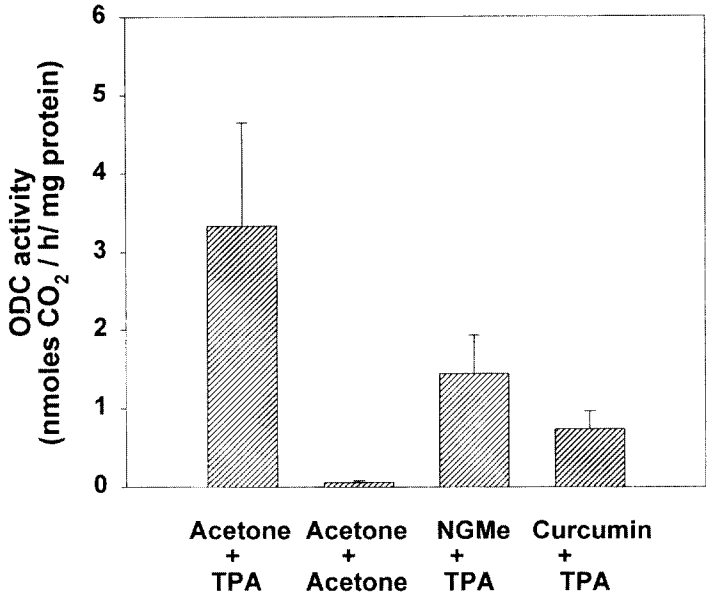

Fig 4. Effects of NgMe pretreatment on TPA-induced epidermal ODC activity in female ICR mice. Animals were treated topically with 0.85 mg NgMe 30 min prior to 5 nmol TPA. Curcumin (20 nmol) was used as a positive reference compound.

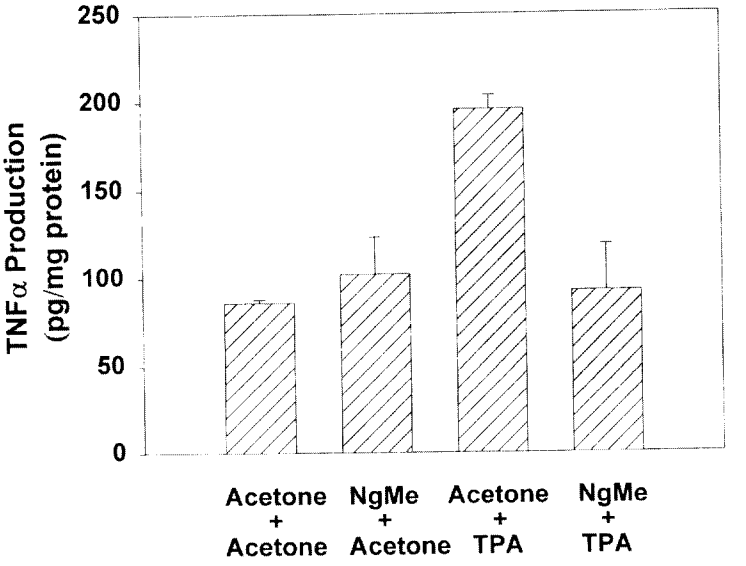

Fig 5. Suppression of TNFα production in mouse skin by NgMe. NgMe (0.682 mg) was topically applied to the shaven backs of female ICR mice 30 min prior to TPA (10 μg) in acetone. Levels of TNFα were determined 5 h later as described in Materials and Methods.

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