Antioxidant and Antipromotional Effects of the Soybean Isoflavone Genistein (43844)

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Abstract. **Antioxidant and antipromotional effects of the soybean isoflavone genistein have been studied in HL-60 cells and the mouse skin tumorigenesis model. Effects of structure-related flavone/isoflavones on hydrogen peroxide (H_2O_2) production by 12-O-tetradecanoylphorbol-13-acetate (TPA)-activated HL-60 cells and superoxide anion ($O_2^{\cdot-}$) generation by xanthine/xanthine oxidase were compared. Of tested isoflavones, genistein is the most potent inhibitor among TPA-induced H_2O_2 formation by (dimethyl sulfoxide) DMSO-differentiated HL-60 cells, daidzein is second, and apigenin and biochanin A show little effect. In contrast, genistein, apigenin, and prunectin are equally potent in inhibiting $O_2^{\cdot-}$ generation by xanthine/xanthine oxidase, with daidzein showing a moderate inhibitory effect and biochanin A exhibiting no effect. These results suggest that the antioxidant properties of isoflavones are structurally related and the hydroxy group at Position 4′ is crucial in both systems. Dietary administration of 250 ppm genistein for 30 days significantly enhances the activities of antioxidant enzymes in the skin and small intestine of mice. Further studies show that genistein significantly inhibits TPA-induced proto-oncogene expression (c-*fos*) in mouse skin in a dose-dependent manner. In a two-stage skin carcinogenesis study, low levels of genistein (1 and 5 μmol) significantly prolong tumor latency and decrease tumor multiplicity by approximately 50%. We conclude that genistein's antioxidant properties and antiproliferative effects may be responsible for its anticarcinogenic effect. Its high content in soybeans and relatively high bioavailability favor genistein as a promising candidate for the prevention of human cancers.** [P.S.E.B.M. 1995, Vol 208]

Epidemiological studies show that consumption of soybean-containing diets have been associated with the lower incidence of certain human cancers in Asian compared with Caucasian populations (1–3). Animal experiments suggest that soybean diets inhibit radiation- and chemical-induced tumors of the mammary (2, 4), skin (5), and liver (6). Although several studies attributed the anticarcinogenic effect of soybeans to protease inhibitors, a study conducted by Barnes *et al.* (2) showed that after inactivation of protease inhibitors by autoclaving, soy diets still strongly inhibited carcinogen-induced mammary tumors in rats. This observation led to the hypothesis that soybean isoflavones may be responsible for the anticarcinogenic effect of soy. Genistein is the most abundant isoflavone in soy and has been identified as a potent inhibitor of protein tyrosine kinases (PTK) *in vitro* (7). Since tyrosine phosphorylation plays a crucial role in cell proliferation and transformation, genistein may have important anticancer properties. Genistein has been shown to selectively inhibit the growth of ras oncogene-transfected NIH 3T3 cells (8), and diminish platelet-derived growth factor-induced c-*fos* and c-*jun* expression in CH310T1/2 fibroblasts (9). In addition, genistein inhibits DNA topoisomerase II (10) and ribosomal S6 kinase (11), which may lead to protein-linked DNA strand breaks and tumor cell growth arrest.

Genistein also exhibits antioxidant properties by preventing hemolysis of red blood cells by dialuric

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acid or H_2O_2 (12, 13), and protecting microsomal lipid peroxidation induced by an Fe^{2+} – ADP complex (14). Reactive oxygen species (ROS) are known to play an important role in mutagenesis and carcinogenesis (15). Production of H_2O_2 and the oxidative modification of certain macromolecules such as DNA bases have been observed in cells and tissues exposed to a variety of tumor promoting agents (16–18). Recently, we have shown that genistein significantly inhibits phorbol ester-type tumor promoter-induced H_2O_2 formation both *in vitro* and *in vivo* (19). In the present study, we have further characterized the antioxidant and antipromotional properties of genistein in cell culture as well as a well-defined multistage carcinogenesis model. Elucidation of genistein's antioxidant properties and its relationship to tumor promotion will not only help us understand the mechanism(s) of genistein's action, but also contribute to application of this soybean isoflavone to the prevention of human cancers.

Material and Methods

Chemicals and Reagents. Genistein (purity >98%) was purchased from LC Laboratories, Woburn, MA. Apigenin, biochanin A, daidzein, and prunectin were purchased from Research Plus Inc. (Bayonne, NJ). Phenol red, horseradish peroxidase (HRPO), H_2O_2 (30%), catalase, superoxide dismutase (SOD), NADPH, oxidized and reduced forms of glutathione, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and dimethyl sulfoxide (DMSO) were purchased from Sigma Co. (St. Louis, MO). RPMI-1640 cell culture medium and fetal bovine serum were purchased from GIBCO (Grand Island, NY).

HL-60 Cell Culture and Differentiation. The HL-60 human leukemia cell line was obtained from American Type Culture Collection (Rockville, MD) and suspended in RPMI-1640 medium supplemented with 20% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, 25 m*M* HEPES buffer, and 100 units/ml penicillin and streptomycin. Cells were cultured in a humidified atmosphere at 37°C in 5% CO_2. In order to induce myeloid differentiation, HL-60 cells were cultivated in medium containing 1.3% DMSO for 7 days. This procedure was shown to produce more than 90% mature cells (neutrophil-like leukocytes) (19). The mature cells were harvested by centrifugation and washed twice with Krebs-Ringer phosphate buffer containing 5 m*M* glucose (KRPG) to remove excess DMSO. Cell pellets were resuspended in KRPG buffer and cell numbers counted using a hemocytometer.

Treatment of Animals. Female CD-1 mice, 6–7 weeks old, were purchased from Charles River Laboratories (Wilmington, MA), and kept under standard conditions (12:12-hr light:dark cycle, humidity 50% ± 15%, temperature 22° ± 2°C and 12 air changes/hr). Food and water were provided *ad libitum*. The dorsal hair of the mice was shaved with surgical clippers 48 hr prior to experiments. Only those at hair-growth resting phase were used for the experiments. All reagents were topically applied to mouse dorsal skin in 0.2 ml acetone. Mice were sacrificed by cervical dislocation and skins removed for analyses of proto-oncogene expression. The tumorigenesis study was conducted as previously described (20).

Analyses of H_2O_2 Formation. The amount of H_2O_2 released by TPA-activated HL-60 cells was measured as previously described (19). Briefly, 2.5×10^5 cells were suspended in KRPG buffer containing 100 μg phenol red and 50 μg HRPO/ml. Different concentrations of isoflavones were added to the reaction mixture and pre-incubated with cells at 37°C for 15 min, then 25 n*M* TPA was added, and the incubation continued for another 30 min. Catalase (5 μg/ml) was added to terminate the reaction, the pH adjusted to 12.5 with 1 *N* NaOH, and the cells removed by centrifugation. The amount of phenol red oxidized by H_2O_2 in the medium was determined spectrophotometrically at 610 nm based on a standard curve of HRPO-mediated oxidation of phenol red. The results were expressed as nmol of $H_2O_2/2.5 \times 10^5$ cells.

Measurement of $O_2^{\cdot -}$ Production. The SOD-inhibitable reduction of cytochrome c assay was used to measure $O_2^{\cdot -}$ production by xanthine/xanthine oxidase as described by O'Brien (21) with a slight modification. All experiments were conducted in duplicate in a final volume of 1 ml of reaction mixture containing different concentrations of test compounds (in 10 μl of DMSO), 2.5 m*M* xanthine, and 0.3 m*M* ferricytochrome c in 50 m*M* phosphate buffer (pH 7.4). The reaction was initiated by adding 0.1 unit of xanthine oxidase, and $O_2^{\cdot -}$ production was quantitated by measuring absorbance alterations at 550 nm.

Assays for Antioxidant Enzymes. Mice were fed AIN-76A diet containing 250 ppm genistein for 30 days and sacrificed at the end of the experiment. Liver, lung, small intestine, kidney, and skin were collected for analyses of catalase (22), SOD (23), glutathione peroxidase (GSH-Px) (23), and glutathione reductase (GSSG-R) (24).

Analyses of c-*fos* and c-*jun* by Northern Hybridization. PolyA RNA was isolated from mouse skin using an oligo-dT column. A total of 3 μg mRNA was denatured by incubation at 55°C for 10 min and loaded onto an agarose/formaldehyde gel. After electrophoresis, RNA in the agarose gel was transferred to a nitrocellulose membrane for hybridization. The cDNA probes of c-*fos*, c-*jun*, and cyclophilin were prepared using the nick translation technique. The hybridized filter was washed and autoradiography visualized by exposing the filter to x-ray film at −70°C.

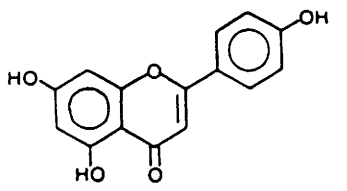
C-*fos*, c-*jun*, and cyclophilin RNA was quantitated using densitometry.

Tumor Promotion Study. Five groups of CD-1 female mice (15/group) were used in the experiment. Each group was challenged with an initiation (one time only)/prevention/promotion protocol (TPA in acetone vehicle). The time between prevention and promotion was 30 min with a regimen of two treatments per week. Group 1 (negative control) was treated with an acetone/acetone/acetone procedure. Groups 2–5 were initiated with 100 nmol of 7,12-dimethylbenz(a)anthracene (DMBA), followed by application of acetone/1 μmol genistein (Group 2—genistein control), acetone/8.5 nmol TPA (Group 3—positive control), and 1 or 5 μmol genistein/8.5 nmol TPA (Group 4 and 5—genistein intervention). Genistein (or acetone)/TPA treatment continued for 17 weeks.

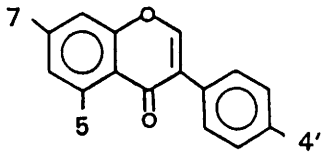
Results

Effect of Structure-Related Isoflavones on H_2O_2 Production by TPA-Activated HL-60 Cells. HL-60 cells are a continuous cell line derived from a patient with promyelocytic leukemia. These cells can be differentiated by DMSO treatment in culture to produce H_2O_2 in response to the tumor promoter TPA. Our previous studies (19) show that under optimal conditions, TPA stimulated HL-60 cells to produce comparable levels of H_2O_2 to those produced by human polymorphonuclear leukocytes (PMN). Using this cell line, we determined the effect of several structurally related flavone/isoflavones on H_2O_2 production by TPA-activated HL-60 cells. Figure 1 shows the structures of flavone/isoflavones tested in the experiment. Figure 2 indicates that genistein is the most potent inhibitor of TPA-stimulated H_2O_2 by HL-60 cells among the tested compounds, daidzein is second, and apigenin and biochanin A show only weak activity even at high concentrations. These results suggest that inhibition of cell-derived H_2O_2 production by genistein might be enhanced by the presence of the hydroxy groups at Positions 4′ and 5 and the second aromatic ring at the Position C-3.

Effect of Structure-Related Isoflavones on $O_2^{\cdot -}$ Production by Xanthine/Xanthine Oxidase. We further determined the effect of flavone/isoflavones on $O_2^{\cdot -}$ generation by xanthine/xanthine oxidase. Figure 3 shows that genistein is the most potent inhibitor of $O_2^{\cdot -}$ formation with apigenin and prunectin second, daidzein exhibiting only moderate inhibition, and biochanin A having no effect. This experiment suggests that the hydroxy group at Position 4′ might play a key role in suppressing $O_2^{\cdot -}$ generation by xanthine/xanthine oxidase and that substitution at C-7 might be of minor influence. Loss of the hydroxy group at this position totally diminishes inhibitory effects whereas replacement of the hydroxy group at other positions does not, or only slightly, affects $O_2^{\cdot -}$ generation. Genistein-mediated inhibition of $O_2^{\cdot -}$ generation by xanthine/xanthine oxidase is more potent than inhibition of cell-derived H_2O_2 production. At a concentration of 20 μ*M* genistein, $O_2^{\cdot -}$ generation was com-



Apigenin



Isoflavone

	Hydroxy Groups		
	5	7	4′
Genistein	-OH	-OH	-OH
Biochanin A	-OH	-OH	$-COH_3$
Daidzein	-H	-OH	-OH
Prunectin	-OH	$-COH_3$	-OH

Figure 1. Structure of tested flavone/isoflavones.

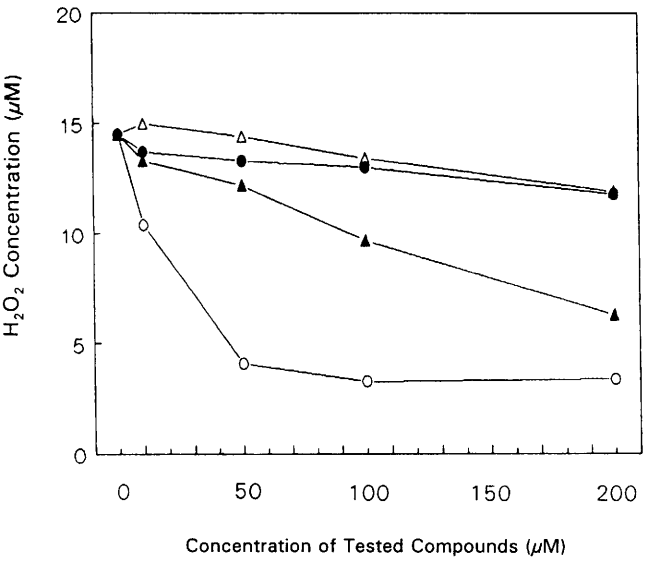


Figure 2. Effect of flavone/isoflavones on H_2O_2 production by TPA-activated HL-60 cells. Assays for cell-derived H_2O_2 are from two experiments with each assay performed in triplicate. Results are expressed as the amount of H_2O_2 (μ*M*) released by TPA-activated HL-60 cells, which were incubated with genistein (○), apigenin (△), biochanin A (●), or daidzein (▲). Standard errors for all determinations are less than 10% of the mean and not shown in the figure.

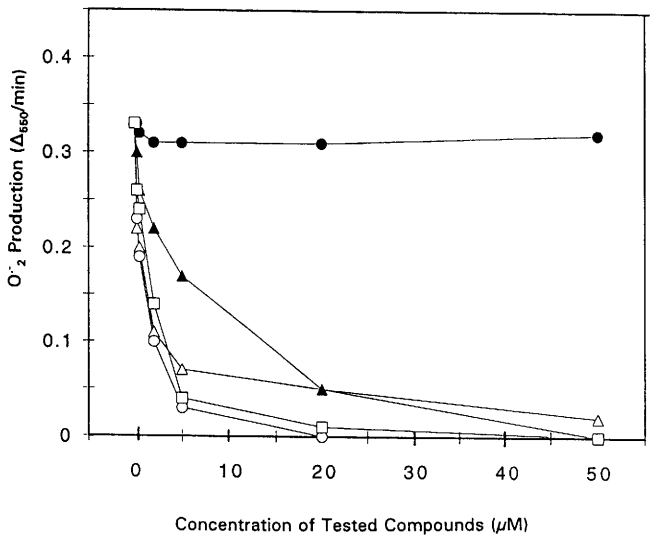


Figure 3. Effect of flavone/isoflavones on $O_2^{\cdot -}$ production by xanthine/xanthine oxidase. Assays for $O_2^{\cdot -}$ production are from at least three experiments with each assay performed in duplicate. Rate of formation of oxidized ferricytochrome c was measured in a reaction mixture incubated with genistein (○), apigenin (△), biochanin A (●), daidzein (▲) or prunectin (□). Standard errors for all determinations are less than 10% of the mean and not shown in the figure.

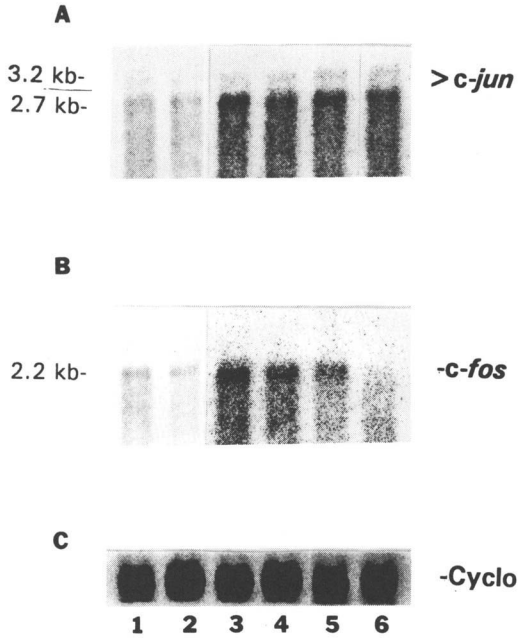


Figure 4. Effect of genistein on TPA-induced c-*fos* and c-*jun* expression. Mice were topically treated with acetone vehicle or genistein 30 min before application of 8.5 nmol TPA (except for controls), and sacrificed 2 hr after TPA treatment. Skin was removed and mRNA purified. Proto-oncogene expression was analyzed by Northern hybridization. (A) c-*jun;* and (B) c-*fos;* and (C) cyclophilin. (Lane 1) acetone/acetone; (Lane 2) acetone/5 μmol genistein; (Lane 3) acetone/TPA; (Lane 4) 1 μmol genistein/TPA; (Lane 5) 5 μmol genistein/TPA; and (Lane 6) 10 μmol genistein/TPA.

pletely suppressed, whereas inhibition of cell-derived H_2O_2 plateaus at 100 μ*M* genistein, with a maximum efficacy of approximately 80%.

Effect of Dietary Genistein on the Activities of Antioxidant Enzymes. Mice were fed 250 ppm genistein in diet for 30 days and several organs were removed for analyses of antioxidant enzymes. Table I shows that dietary administration of genistein significantly increases antioxidant enzyme activities in skin and small intestine. Although enzyme activities were also moderately elevated in other tissues such as liver, kidney, and lung, no statistically significant differences were observed (data not shown).

Effect of Genistein on TPA-Induced Proto-oncogene Expression In Mouse Skin. The tumor promoter TPA has been known to induce overexpression of proto-oncogene in mouse skin (25, 26). Using mouse skin as a model, we determined the effect of genistein on TPA-induced c-*fos* and c-*jun* expression *in vivo*. Figure 4 shows that topical application of a promoting dose (8.5 nmol) of TPA significantly induces expression of c-*fos* and c-*jun* mRNA in mouse skin (Lane 3) compared with acetone-treated control (Lane 1). As reported by Zwiller *et al.* (9), there are two c-*jun* mRNA fragments (2.7 and 3.2 kb) due to the presence of two polyadenylation signals. Densitometry quantitation indicates that TPA significantly increased expression of these proto-oncogenes by 1.7- (c-*jun* 3.2 kb), 3.2- (c-*jun* 2.7 kb), and 7.0-fold (c-*fos*), as compared with the acetone-treated control. Treatment of mouse skin with genistein alone slightly decreased the basal levels of c-*fos* and c-*jun* mRNA (Lane 2). However, pretreatment of mouse skin with genistein suppressed TPA-induced expression of c-*fos* with little effect on c-*jun* (Lane 4–6). At a dose of 10

Table I. Effect of Genistein on the Activities of Antioxidant Enzymes[a]

	Catalase	SOD	GSH-PX	GSSG-R
Skin				
Control	1.4 ± 0.07	16.1 ± 0.7	2.6 ± 0.1	0.8 ± 0.03
Genistein	1.6 ± 0.06	19.0 ± 1.1[b]	2.9 ± 0.1[b]	0.9 ± 0.02[b]
Intestine				
Control	1.1 ± 0.04	40.4 ± 4.2	7.2 ± 0.2	4.9 ± 0.2
Genistein	1.4 ± 0.11[b]	39.0 ± 2.6	7.0 ± 0.1	6.1 ± 0.4[b]

[a] Data represent mean ± SE of assays in duplicate from nine mice/group. Catalase expressed as μmol H_2O_2 consumed/min/mg tissue; SOD as μg SOD/g tissue; GSH-Px and GSSG-R as μmol NADPH oxidized/min/g tissue.
[b] Statistically significant versus control by two-tailed Student's *t* test, $P < 0.05$.

μmol genistein, TPA-induced c-*fos* expression was almost completely inhibited.

Effect of Genistein on TPA-Promoted Skin Tumorigenesis. The mouse skin tumorigenesis experiment shows that genistein is a potent antipromoter in the two-stage carcinogenesis model. Figure 5 shows that genistein significantly prolongs tumor latency periods and decreases tumor multiplicity in CD-1 mice initiated with 100 nmol DMBA and promoted with 8.5 nmol TPA. At 17 weeks post-treatment of tumor promoter, low levels of genistein (1 and 5 μmol) inhibited tumor multiplicity by approximately 36% and 46%, respectively, without obvious toxicity as determined from body weight and food consumption (data not shown). Latency of tumors was prolonged by approximately three weeks in genistein-pretreated mice. Tumor incidence for the genistein treated groups were significantly below those of positive controls up to week 12 of the experiment (approximately 45% in both genistein treated groups compared to 80% for positive controls); however by the end of the experiment, while still below positive controls, these reductions were no longer statistically significant (data not shown). This is perhaps attributable to a genistein-mediated increase in latency.

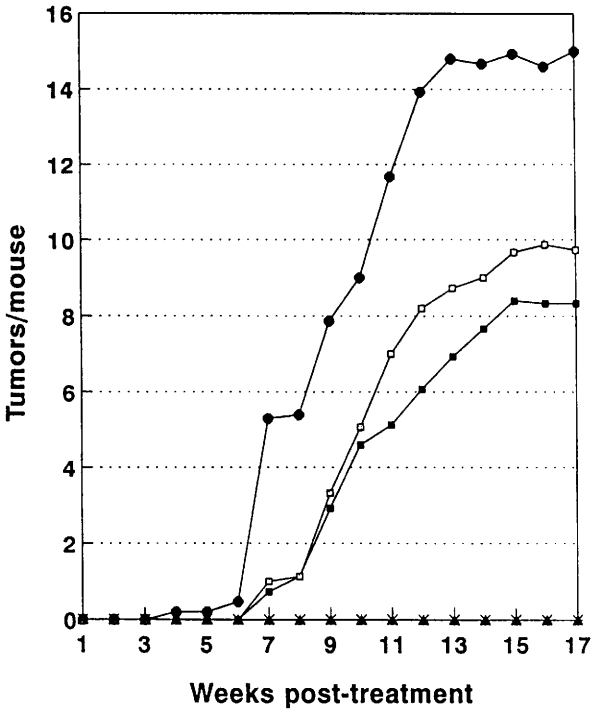


Figure 5. Effect of genistein on tumor multiplicity. Five groups of mice (15/group) were used in the experiment. A negative control group (✱) was treated with an acetone/acetone/acetone procedure. All remaining mice were initiated with 100 nmol DMBA. Genistein control (△) was followed by twice-weekly acetone/1 μmol genistein, positive control (●) by acetone/8.5 nmol TPA, and genistein intervention groups by 1 μmol (□) and 5 μmol (■) genistein/8.5 nmol TPA, respectively. Time between prevention and promotion treatments is 30 min. Genistein (or acetone)/TPA treatment continued for 17 weeks.

Discussion

Genistein has drawn wide attention in recent years because of its potent inhibitory effect on tyrosine protein kinases (7). A number of studies have shown that genistein inhibits tumor cell growth, suppresses oncogene expression, and induces cell differentiation of malignant cells (8–11). Although most studies have attributed the anticancer properties of genistein to its inhibition of signal transduction, we hypothesize that genistein may also exert its anticarcinogenic effects through antioxidant mechanism(s). In the present study, we have examined the effect of genistein as an antioxidant on tumor promoter-induced biochemical events and tumorigenesis.

We previously reported that genistein strongly inhibited TPA-induced H_2O_2 production in human PMNs and HL-60 cells with a minimal effect on cellular $O_2^{\bullet -}$ production. In the present study, we compared the effect of genistein and its structurally related flavone and isoflavones on TPA-induced H_2O_2 production by HL-60 cells. The results show that genistein is the most potent inhibitor of TPA-stimulated H_2O_2 by HL-60 cells of the tested compounds. The antioxidant potencies of isoflavones are structurally related and closely associated with the presence of hydroxy groups at Position 4′ and 5 and the position (C-3 versus C-2) of the aromatic ring. Suppression of $O_2^{\bullet -}$ production by xanthine/xanthine oxidase is also structurally related, but different from the effects on cell-derived H_2O_2 generation since most tested flavone/isoflavones except biochanin A exhibit an inhibitory effect on $O_2^{\bullet -}$ production. Apigenin, which has no effect on H_2O_2 production by TPA-activated HL-60 cells, exhibits a potency in the inhibition of $O_2^{\bullet -}$ production that is almost equal to that of genistein. Suppression of $O_2^{\bullet -}$ production by isoflavones may be due to their inhibitory effect on xanthine oxidase activity rather than a direct scavenging effect on $O_2^{\bullet -}$ *per se* because a previous study by this laboratory shows that genistein has a very weak effect on $O_2^{\bullet -}$ released by TPA-activated HL-60 cells (19). Our results show that dietary administration of genistein can modulate the activities of antioxidant enzymes. The activities of catalase, SOD, GSH-Px, and GSSG-R were determined in mouse skin, small intestine, liver, kidney, and lung of mice fed 250 ppm genistein in diet for 30 days. The activities of most antioxidant enzymes were significantly enhanced in skin and small intestine whereas only slight effects were observed in other organs. Our previous study shows that genistein strongly inhibits TPA-induced oxidant formation in mouse skin (19). TPA is known to induce H_2O_2 production by phagocytic cells and epidermal cells (27), increase xanthine oxidase activity (28), and diminish antioxidant enzyme activities (29).

Therefore, it is possible that genistein counteracts the promotional effects of TPA through inhibiting oxidant formation by epidermal cells and epidermal xanthine oxidase, and by enhancing antioxidant enzyme activities in skin.

Genistein has been shown to downregulate TPK activities induced by several activators, such as normal growth factors (e.g., EGF and PDGF) and viral oncogene products (e.g., $pp^{60\ v\text{-}src}$ and $pp^{110\ gag\text{-}fes}$) (7). This anti-TPK activity of genistein is considered to be responsible for the inhibition of ras-transfected NIH 3T3 cell growth (8) and suppression of PDGF-induced expression of c-*fos* and c-*jun* in fibroblasts (9). The regulation of PDGF-induced c-*jun* and c-*fos* expression may be though phosphorylating serum binding factor (30). An alternative pathway to regulate c-*fos* and c-*jun* expression involves activation of PKC. TPA is a potent protein kinase C (PKC) activator and stimulates expression of c-*fos* and c-*jun in vitro* and *in vivo* by phosphorylating serum binding factor (30). In the case of c-*fos* expression, binding of phosphorylated serum response factor to serum responsive element (SRE) can be mediated by both PKC-dependent and PKC-independent pathways (30). It appears that inhibition of TPA-induced c-*fos* expression by genistein is not a direct effect on the PKC-dependent pathway since genistein is a poor inhibitor of PKC activity with an apparent IC_{50} >100 μg/ml (7). We assume that induction of immediate early gene expression depends on the balance of several protein kinases. Recently, mitogen-activated protein (MAP) kinases have been identified as an integration point for multiple biochemical signals (31, 32). Phorbol esters can activate the MAP kinases to phosphorylate substrates consisting of tyrosine and threonine. We speculate that the MAP kinases may be a target of genistein and that genistein's inhibition of tyrosine phosphorylation by MAP kinases decreases the binding activity of serum response factors to the SRE, subsequently inhibiting the TPA-induced c-*fos* expression. Stevenson *et al.* recently reported that x-irradiation, phorbol esters, and H_2O_2 stimulate MAP kinase activity through the formation of ROS (33). Since genistein is an inhibitor of ROS generation, the inhibition of c-*fos* expression might be through suppressing TPA-stimulated MAP kinase activity. In our study, genistein significantly inhibited TPA-induced c-*fos* expression, but had little effect on TPA-induced c-*jun*. One possible explanation may be that interference of genistein on protooncogene expression occurs at different promotional sites since TPA was shown to induce c-*fos* expression via the SRE located on upstream of the gene, whereas induction of c-*jun* by TPA occurs at an AP1 site rather than SRE (30). However, the precise mechanism(s) by which genistein inhibits TPA-induced c-*fos* expression *in vivo* is not clear and requires further study. The potent inhibitory effect on tumor promoter-induced c-*fos* expression suggests that genistein may be a promising antipromotional agent.

Accumulating evidence shows that ROS, and subsequent oxidative modification of macromolecules are involved in many mutagenic and carcinogenic processes (15). Our previous studies show that TPA-mediated oxidative events and oxidative DNA damage can be inhibited by other antitumor agents (17, 18). Since genistein possesses similar antioxidant properties to those antitumor agents, it is possible that genistein can work as a antitumor promoter *in vivo*. Our animal tumorigenesis experiment has confirmed an antipromotional activity of genistein, showing that 1 and 5 μmol genistein, which suppressed TPA-induced H_2O_2 formation and c-*fos* expression in mouse skin, significantly inhibited TPA-promoted skin tumor formation in a two-stage carcinogenesis model. The fact that genistein potently inhibits oxidant formation and proto-oncogene expression suggests that the antioxidant properties and antiproliferative effects of genistein may, at least in part, be responsible for its anticarcinogenic mechanism(s).

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