Effects of Genistein on the Polyamine Metabolism and Cell Growth in DLD-1 Human Colon Cancer Cells

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Abstract: Polyamines and their rate-limiting enzyme, ornithine decarboxylase (ODC), are actively involved in cell growth and differentiation. The phytoestrogen genistein has been demonstrated to possess antitumor properties by influencing proliferation, differentiation, and apoptosis. The aim of this study was to investigate the effects of genistein at concentrations ranging from 0.01 to 100 µM on the polyamine biosynthesis, cell proliferation, and apoptosis in the estrogen receptor-positive DLD-1 human colon cancer cell line. Polyamine levels and ODC activity were evaluated by high performance liquid chromatography and radiometric technique, respectively. The proliferative response was estimated by [³H]-thymidine incorporation and the colorimetric 3-(4,5 di-methylthiazol-2-yl)–2,5-diphenyltetrazolium bromide test. Apoptosis was investigated by DNA fragmentation. Bax and Bcl-2 gene expressions were evaluated by multiplex-polymerase chain reaction. At concentration ≥1 µM, genistein decreased significantly the ODC activity and the polyamine levels. At the same concentration, genistein also increased significantly Bax mRNA expression, but not Bcl-2 mRNA expression. Higher concentrations (≥10 µM) were needed to obtain a significant inhibition of cell proliferation and DNA fragmentation. The results of this study suggest that genistein can affect growth of DLD-1 cells by both decreasing polyamine biosynthesis and inducing apoptosis. However, further studies are required to assess the true ability of a soy rich diet in modifying colon cancer risk.

Introduction

Phytoestrogen genistein, the predominant soy isoflavones, has drawn attention in recent years because of its potential cancer protective effects. There is now sufficient evidence of an association between high consumption of phytoestrogen-rich foods and a low incidence of breast and prostate cancer within populations of Asian countries (1). Besides, it has been speculated that isoflavone-enriched soybean products might also prevent non-hormone-related neoplasms such as colon cancer (2,3). The role of diet is gaining increasing attention because particular foods may contain different biologically active compounds. Therefore, identifying dietary phytoestrogens with antitumor activity and investigating their mechanisms of action may lead to significant advances in the prevention of human cancer.

In vitro experiments have demonstrated that genistein influences proliferation, differentiation, and apoptosis in a variety of tumor cell types (4–7).

Several possible mechanisms for the anticancer effects of genistein have been proposed including inhibition of angiogenesis, topoisomerase, tyrosine kinase activity, and antioxidant properties (8). Moreover, it has been observed that in the rat mammary epithelium, ornithine decarboxylase (ODC) activity and polyamine concentrations were significantly lower in the group treated with soy protein than in control (9).

Polyamines, putrescine, spermidine, and spermine are ubiquitous short-chain aliphatic amines that play an important role in cell proliferation and differentiation (10). Abnormal hyperproliferative cells such as in neoplastic and preneoplastic tissue exhibit very high requirements for polyamines to sustain cell growth through elevated DNA, RNA, and protein synthesis (11). The metabolism of polyamines begins with the ODC, a rate limiting enzyme that is highly regulated in all of the cells and responds to a wide variety of growth promoting stimuli (12).

It is known that genistein is structurally similar to estrogens and produces effects suggestive for estrogenicity (8), being capable of binding to the two subtypes of estrogen receptors (ERs): ERα and ERβ (13). ERs have been found in diverse solid tumors including esophageal, gallbladder, gastric, and colorectal cancer (14–16), all these tumors believed to be nontarget organs for sex hormones. In this connection, we have previously shown both in vivo and in vitro that estrogens can exert an inhibitory effect on gastrointestinal cell proliferation by interacting with growth factors (17,18), apoptotic processes (19), and the metabolism of polyamines (20).

In experimental works, a wide range of genistein concentrations from physiological levels to supraphysiological ones have been used to investigate its property on colonic cell proliferation (6,21–23). Interestingly, it was also proved that different concentrations of genistein can exert a dual effect on cell proliferation of cultured human intestinal cells (24).

Starting from these concepts, we designed this study to investigate the effects of increasing concentrations of genistein on the polyamine biosynthesis and cell growth in the ER-positive, DLD-1 human colon cancer cell line. We also investigated its effects on apoptosis and mRNAs expression of Bax and Bcl-2 apoptosis-related proteins to further elucidate the role of this isoflavone in modulating colon cancer cell growth.

Materials and Methods

Cell Culture Conditions

Human colon adenocarcinoma-derived DLD-1 cells were obtained from the Interlab Cell Line Collection (IST, Genoa, Italy). Cells were routinely cultured in RPMI 1640 without phenol red supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, in monolayer culture, and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. At confluence, the grown cells were harvested by means of trypsinization and serially subcultured with a 1:4 split ratio. All cell culture components were purchased from Sigma-Aldrich (Milan, Italy).

ER Characterization

Before proceeding with experiments based on genistein administration, we evaluated the expression of the ERα and β subtypes in DLD-1 cells in our laboratory by nested polymerase chain reaction (PCR) and Western blotting. In addition, the DLD-1 cells were characterized for the mRNA expression of 5 ERβ isoforms (ERβ 1–5). Nested PCR and Western blotting procedures as well as primer sequences and reverse-transcriptase (RT) PCR conditions were performed as described previously (25).

Genistein Treatment

In the experiments investigating the effect of genistein under estrogen-depleted culture conditions, DLD-1 cells were seeded at a density of 2×10^5 cells/5 ml of phenol red-free RPMI 1640 containing 10% FBS in 60 mm tissue culture dishes (Corning Costar Co., Milan, Italy). After 24 h, to allow for attachment, the medium was removed, and RPMI 1640 containing 5% dextran-coated charcoal-treated FBS was added to cell line.

The cells were incubated for a further 24 h, and then the medium was replaced by fresh culture medium containing 5% charcoal-stripped FBS with genistein at increasing concentrations (0.01 µM, 0.1 µM, 1 µM, 10 µM, 20 µM, 30 µM, and 100 µM) dissolved in dimethyl sulfoxide (DMSO). The cells were allowed to grow for another 24 h and then trypsinized. The cell pellet obtained after low-speed centrifugation was used for the subsequent analyses.

Before proceeding with the experiments, estrogen levels were evaluated in the estrogen stripped medium in which DLD-1 cells were seeded for 24 and 48 h. By using a specific radioimmunoassay, the levels were found below the detection limit.

Each experiment included an untreated control and a control with the equivalent concentration of DMSO as had been used for adding phytoestrogen. The solvent reached a concentration not higher than 0.3% in all experiments. Triplicate cultures were set up for each phytoestrogen concentration and for control, and each experiment was repeated 4 times.

Cell viability, determined using the trypan blue exclusion test, always exceeded 90%.

Polyamine Analysis

For the evaluation of the polyamine levels after genistein treatment, each cell culture pellet was homogenized in 700 µl of 0.9% sodium chloride mixed with 5 µl (174 nmol/ml) of an internal standard (1,10-Diaminodecane).

To precipitate the proteins, 50 µl of perchloric acid 3M were added to the homogenate. After 30 min of incubation in ice, the homogenate was centrifuged for 15 min at 7,000 *g*.

The supernatant was filtered (Millex-HV$_{13}$ pore size 0.45 mm; Millipore, Bedford, MA) and lyophilized.

The residue was dissolved in 250 µl of hydrochloric acid (HCl; 0.1 N). Aliquots (100 µl) were reacted with dansyl chloride, and the dansyl-polyamine derivatives were determined by high performance liquid chromatography as previously described (26).

Polyamine levels were expressed as concentration values in nmol/mg of protein.

ODC Activity

ODC activity was measured with a radiometric technique that estimated the amount of $^{14}CO_2$ liberated from DL-[1-^{14}C]-ornithine (specific activity, 42.47 mCi/mmol; New England Nuclear, Monza, Italy) (27).

The cell culture pellet ($2–4 \times 10^6$ cells) was homogenized in 0.6 ml ice-cold Tris-HCl (15 mM, pH 7.5) containing 2.5 mM dithiothreitol, 40 µM pyridoxal-5′-phosphate, and 100 µM ethylenediaminetetraacetate acid and then centrifuged at 30,000 *g* for 30 min at 4°C.

An aliquot of supernatant (200 µl) was added to a glass test tube containing 0.05 µCi DL-[1-^{14}C]-ornithine and 39 nmol DL-ornithine. After incubation for 60 min at 37°C, the reaction was stopped by adding trichloroacetic acid (TCA) to a final concentration of 50%. $^{14}CO_2$ liberated from DL-[1-^{14}C]-ornithine was trapped on filter paper pretreated with 40 µl NaOH (2N), which was suspended in a center well

above the reaction mixture. Radioactivity on the filter papers was determined by a liquid scintillation counter (model 1219 Rackbeta; LKB-Pharmacia, Uppsala, Sweden). ODC activity was expressed as $pmolCO_2/h/mg$ of protein. Enzymatic activity was found to be linear within the range of 50–600 μg of protein ($r^2 = 0.992$). The intra-assay and interassay variation coefficients (CV%) were 6% and 8%, respectively.

Assessment of Cell Proliferation

After DLD-1 cells had been cultured for 24 h with different concentrations of genistein, the proliferative response was estimated by [3H]-thymidine incorporation and the colorimetric 3-(4,5 di-methylthiazol-2-yl)–2,5-diphenyl-tetrazolium bromide (MTT) test.

To determine DNA synthesis, 0.3 μCi/ml of [methyl-3H]–thymidine (85.50 Ci/mmol; NEN Life Science Products Inc., Boston, MA) was added to triplicate dishes in the last 12 h of genistein treatment.

After incubation, the medium was aspired to remove unincorporated [3H]-thymidine, and the cells were maintained with 0.33 N NaOH for 30 min.

To precipitate and hydrolyze the DNA, the resulting cells were harvested by collection onto tube glass containing 40% TCA with 1.2 N HCl and centrifuged at 3,000 g for 15 min. The precipitated DNA was redissolved in 0.33 N NaOH, and then 250μl were transferred into vials containing 3 ml of scintillation fluid. Incorporation of [3H]-thymidine in DNA was determined by scintillation quantitation in a Rackbeta counter (model 1219; LKB-Pharmacia, Turku, Finland).

To determine cell growth by colorimetric test, MTT stock solution (5 mg/ml in medium) was added to each dish at a volume of one-tenth the original culture volume and incubated for 2 h at 37°C in humidified CO_2. At the end of the incubation period, the medium was removed, and the blue formazan crystals were solubilized with acidic isopropanol (0.1 N HCl in absolute isopropanol).

MTT conversion to formazan by metabolically viable cells was monitored by spectrophotometer at an optical density of 570 nm.

Apoptotic Death Assay

After DLD-1 cells had been cultured for 24 h with genistein at concentrations between 0.01 μM and 100 μM, the cytosolic DNA-histone complexes generated during apoptotic DNA fragmentation were evaluated by cell death detection enzyme-linked immunoabsorbent assay (ELISA) kit (Roche Diagnostics GmbH, Mannheim, Germany) following the supplier's instruction. Each experiment was performed 4 times.

Additional plates treated as previously mentioned were analyzed for cell number with the use of MTT test.

The A_{405} obtained from the DNA fragmentation assay was then normalized for cell number, and the results were compared to those for untreated control. Additionally, DNA frag-

mentation was evaluated by gel electrophoresis as described by Cifone et al. (28).

Bax and Bcl-2 mRNA Detection

After treatment of DLD-1 cells with increasing genistein concentrations from 0.01 μM to 100 μM, the mRNAs of Bax and Bcl-2 apoptosis-related proteins were evaluated as following.

Total RNA was extracted by TRI Reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions, spectrophotometrically quantitated, and stored at –80°C in water until analysis.

Then, 2 mg of total RNA were reverse-transcribed separately for target genes (Bcl-2 and Bax) and β-actin, the latter used as the control gene. The β-actin gene was selected as the control gene because its expression is not modified in cells treated by hormone steroids (29).

After RT, the Bax and Bcl-2 cDNA were coamplified with β-actin cDNA (diluted 1:3) in a final volume of 50 μl following the PCR multiplex method described by Henegariu et al. (30).

The thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 min, then 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

PCR products were run on a 2% agarose gel to confirm the presence of bands with the expected size (287bp, 304bp, and 479bp for Bcl-2, Bax, and β-actin, respectively).

For each sample, the mRNA value was determined by ratio between the fluorescent signal of the target gene (Bax or Bcl-2) and that of the β-actin.

The primers sequences were:

5′-GTGGAGCTCTTCAGGGA-3′ (sense), 5′-AGGCAC CCAGGGTGATGCAA-3′ (antisense) for Bax;
5′-CAGGATGCGTCCACCAAGAA-3′ (sense), 5′-GCT CCCGGAGGAAGTCCAAT-3′ (antisense) for Bcl-2; and
5′-GCGGGAAATCGTGCGTGACATTAAGGAGA-3′ (sense), 5′-CGTCATACTCCTGCTTGCTGATCCACATCT GC-3′ (antisense) for β-actin.

The RT and PCR reagents were purchased from Invitrogen (Milan, Italy).

Statistical Analysis

Due to the nonnormal distribution of the data, nonparametric tests were performed. For ODC activity, polyamine levels, and proliferative characteristics of DLD-1 cells, the significance of differences between the groups was determined by Kruskal–Wallis analysis of variance. Comparisons between each single treatment group were analyzed by Wilcoxon Mann–Whitney test. Correlations between single and total polyamine content and increasing genistein concentrations were analyzed by Spearman rank correlation. All data are expressed as mean ± SD. Differences were considered significant at $P < 0.05$. A specific statistical package for

exact nonparametric inference package (STATA Version 6.0 statistical software; Stata Corporation, College Station, TX) was used.

Results

Expression Profiling of ERs

At basal conditions, nested PCR showed that DLD-1 cells express ERβ mRNA but lack ERα mRNA (see Fig. 1A). Western blotting analysis confirmed this finding (Fig. 1B). The DLD-1 cells were also characterized for their ERβ

isoforms. These cells express only the wild type ERβ 1 isoform (Fig. 1C).

Effects of Genistein on Polyamine Biosynthesis

The effects of genistein on the ODC activity were studied at concentrations between 0.01 μM and 100 μM. As shown in Fig. 2, genistein treatment affected the enzymatic activity in DLD-1 cells. Specifically, starting from 1 μM, genistein administration reduced significantly ODC activity compared to untreated cells ($P < 0.01$).
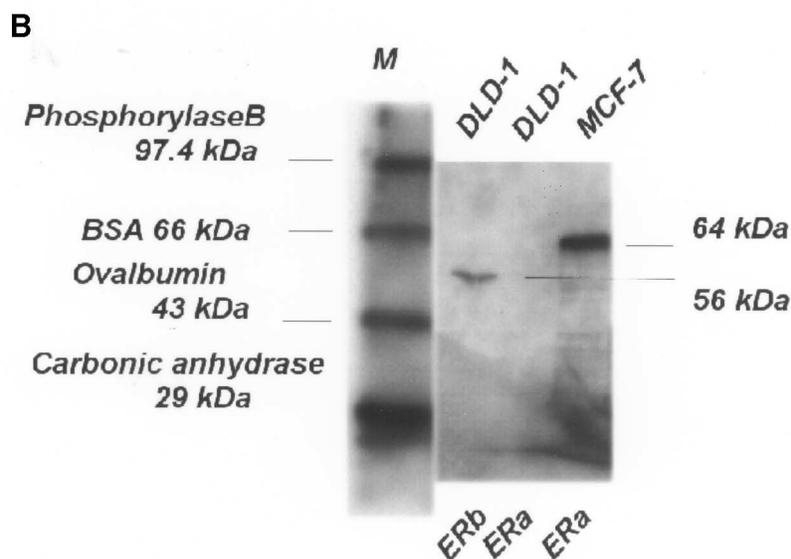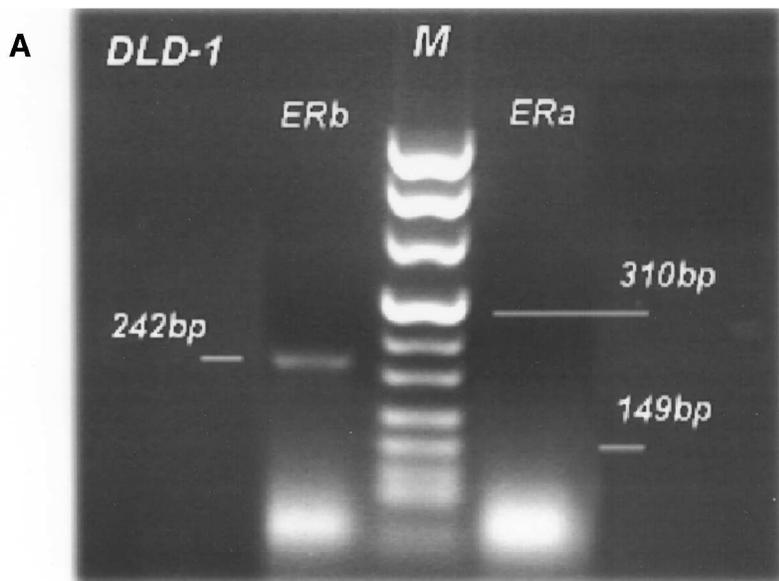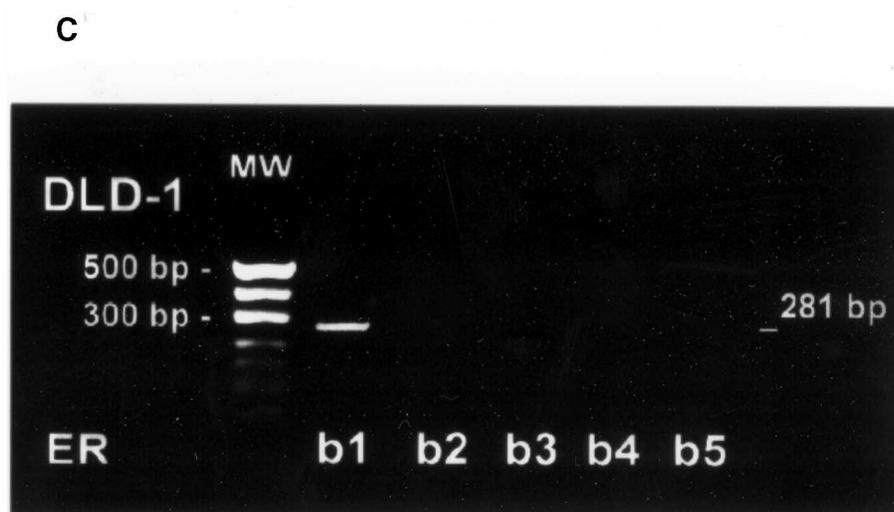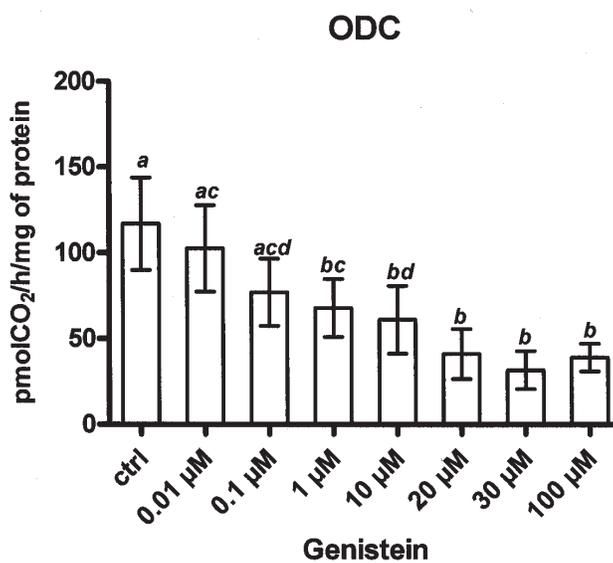

Figure 1. (Continued)

Figure 1. Estrogen receptor characterization in DLD-1 cells. A: reverse-transcriptase polymerase chain reaction. B: Western blotting analysis. C: mRNA expression of 5 estrogen receptors (ER)β isoforms.

Control	0.01μM	0.1μM	1 μM	10 μM	20μM	30μM	100μM
116.8±26.9	102.5±25.0	77.0±19.7	67.8±16.9	61.0±19.8	41.0±14.6	31.7±11.1	39.0±8.1

Figure 2. The effects of genistein on the ornithine decarboxylase (ODC). Genistein was administered at concentrations between 0.01 μM and 100 μM. ODC activity is reported as pmol/CO_2/h/mg of protein (mean ± SD). All results represent the mean of four different experiments. For each genistein concentration, mean values not sharing a common superscript (a, b, c, d) differ significantly ($P < 0.01$).

ODC activity at concentrations equal to or higher than 10 μM were significantly lower than that observed in control and 0.01μM ($P < 0.01$). Besides, with genistein concentrations equal to or higher than 20 μM, differences in ODC activity were significant ($P < 0.01$) also compared to 0.1 μM.

As concerns the polyamine profile, the administration of increasing concentrations of genistein (namely, from 0.01 μM to 100 μM) led to a decrease of the single and total polyamine contents in the DLD-1 cells. The decrease was significant ($P < 0.05$) at concentrations equal to or higher than 1 μM for putrescine, spermidine, and the spermidine:spermine ratio compared to both control cells and cells treated with the lowest genistein concentration (0.01 μM). In cells treated with genistein concentrations equal to or higher than 20 μM, putrescine, spermidine, and the spermidine:spermine ratio were also significantly ($P < 0.05$) lower than that observed at 0.1 μM.

Table 1. The Effects of Increasing Concentrations of Genistein on the Polyamine Content of DLD-1 Cells[a]

	Control	0.01 µM	0.1 µM	1 µM	10 µM	20 µM	30 µM	100 µM
Putrescine	0.06 ± 0.02[*]	0.06 ± 0.02[*]	0.04 ± 0.01[†]	0.03 ± 0.01[‡]	0.03 ± 0.001[‡]	0.03 ± 0.005[§]	0.03 ± 0.005[§]	0.03 ± 0.005[§]
Spermidine	2.75 ± 0.89[*]	2.53 ± 0.99[*]	1.70 ± 0.32[†]	1.47 ± 0.31[‡]	1.54 ± 0.44[‡]	1.25 ± 0.27[§]	1.14 ± 0.079[§]	1.26 ± 0.14[§]
Spermine	6.70 ± 2.05[*]	6.35 ± 1.80[*]	5.30 ± 1.43[*]	5.17 ± 0.93[*]	5.50 ± 0.56[*]	5.47 ± 1.05[*]	5.30 ± 0.86[*]	4.40 ± 1.12[*]
Total Polyamines	9.51 ± 2.91[*]	8.94 ± 2.74[*]	7.04 ± 1.73[**]	6.58 ± 1.10[**]	7.07 ± 0.92[**]	6.74 ± 1.2[**]	6.46 ± 0.85[**]	5.69 ± 1.20[§]
Ratio	0.41 ± 0.06[*]	0.39 ± 0.08[*]	0.33 ± 0.04[†]	0.29 ± 0.06[‡]	0.28 ± 0.06[‡]	0.23 ± 0.03[§]	0.22 ± 0.04[§]	0.29 ± 0.06[§]

a: For each concentration, mean values not sharing a common superscript ([*], [†], [‡], [§], [**]) differ significantly ($P < 0.05$). All data represent the mean ± SD of four consecutive experiments. Polyamines are expressed as nmol/mg protein. Ratio, Spermidine:Spermine.

Genistein at 100 µM reduced significantly ($P = 0.026$) the total polyamine content compared to both control cells and cells treated with 0.01 µM (Table 1).

Finally, the polyamine content resulted to be inversely correlated to genistein concentrations. The significant and inverse relationship between genistein concentrations and the total polyamine content in the DLD-1 cells is reported in Fig. 3 ($r = –0.8810$, $P = 0.0072$; Spearman correlation coefficient). A significant inverse correlation was also present for the spermidine content (data not shown).

Effects of Genistein on DLD-1 Cells Proliferation

Exposure of DLD-1 cell line to increasing concentrations of genistein (from 0.01 µM to 100 µM) under estrogen-depleted culture conditions showed an evident antiproliferative action.

Figure 4 shows the effects of increasing concentrations of genistein on the incorporation of [3H]-thymidine in DNA of DLD-1 cells. At the lowest concentrations (0.01 µM, 0.1 µM, and 1 µM), genistein did not significantly affect [3H]-thymidine incorporation in DNA of cells. Concentrations equal to or higher than 10 µM of the isoflavone caused a significant ($P < 0.01$) reduction in the [3H]-thymidine incorporation compared with both untreated control cells and cells treated with the lowest genistein concentrations (0.01 µM and 0.1 µM). Besides, [3H]-thymidine incorporation in cells

treated with 30 µM and 100 µM differed significantly ($P < 0.05$) also compared to 1 µM.

Figure 5 shows the effect of increasing concentrations of genistein on the conversion of MTT tetrazolium in DLD-1 cells. Concentrations of genistein from 10 µM to 100 µM caused a significant ($P < 0.01$) reduction in conversion of the MTT tetrazolium salt compared with both untreated control cells and cells treated with 0.01 µM genistein. By administering the highest genistein concentrations (30 µM and 100 µM), MTT conversion was reduced significantly ($P < 0.05$) also compared to the lower concentrations 0.1 µM and 1 µM.

Effects of Genistein on Apoptosis

Figure 6 shows the effects of increasing genistein concentrations (from 0.01 µM to 100 µM) on the apoptosis of DLD-1 cells evaluated by enzyme-linked immunoabsorbent assay (ELISA).

Concentrations of genistein between 0.01 µM and 1 µM did not significantly affect DNA fragmentation. On the contrary, DNA fragmentation significantly increased ($P < 0.05$) in cells treated with concentrations of genistein equal to or higher than 10 µM compared to control cells and cells treated with the lowest concentrations (0.01 µM and 0.1 µM). Administration of the highest genistein concentrations (30 µM
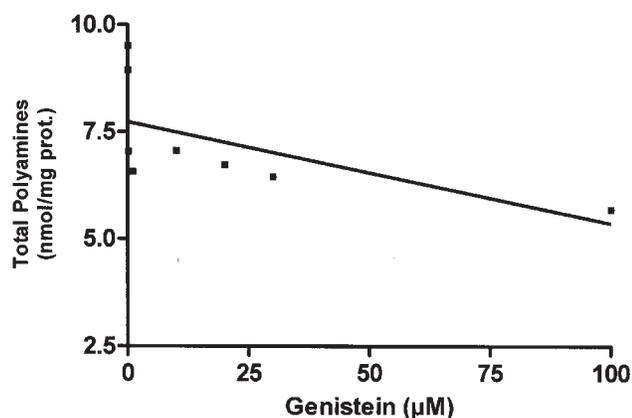

Figure 3. The significant and inverse relationship between genistein concentrations and the total polyamine content in the DLD-1 cells ($r = –0.8810$, $P = 0.0072$).
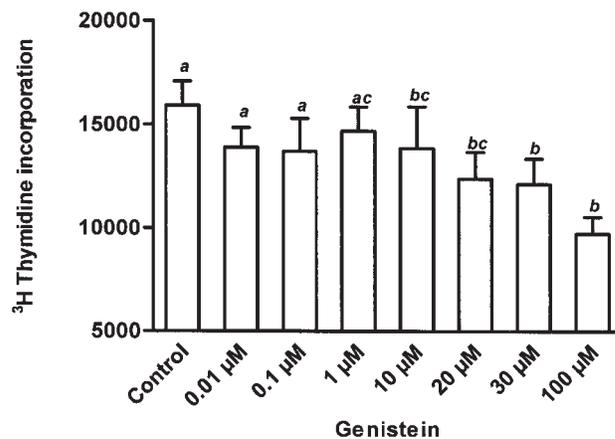

Figure 4. Effects of increasing concentrations of genistein on the incorporation of [3H]-thymidine in DNA of DLD-1 cells. All results represent the mean of four different experiments. For each genistein concentration, mean values not sharing a common superscript (a, b, c) differ significantly ($P < 0.05$).
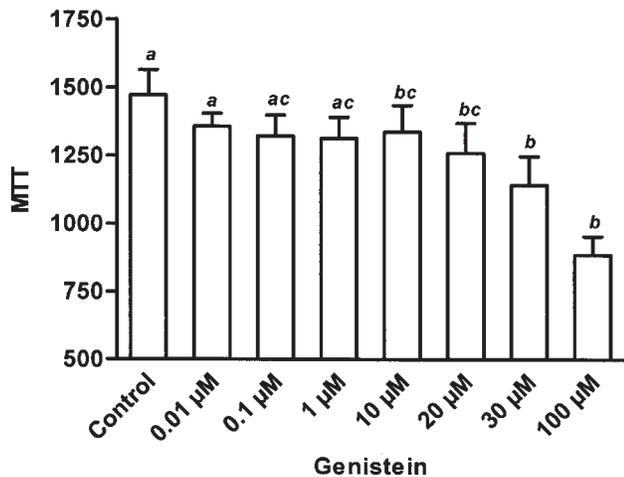
Figure 5. Effects of increasing concentrations of genistein on the in vitro cell growth as measured by the conversion of 3-(4,5 dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide (MTT) tetrazolium salt. All results represent the mean of four different experiments. For each concentration of genistein, mean values not sharing a common superscript (a, b, c) differ significantly ($P < 0.05$).
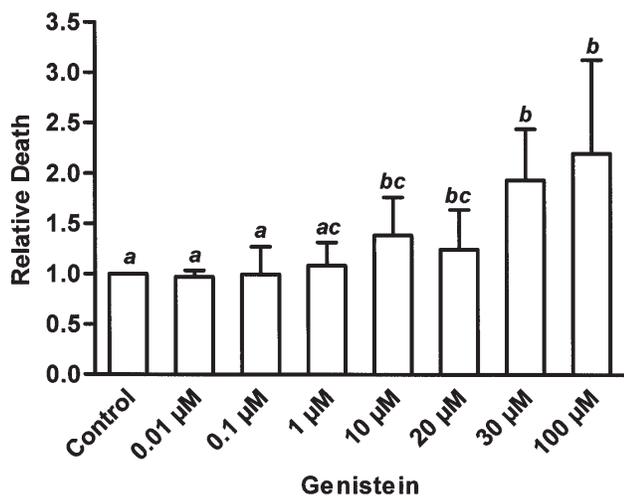

Figure 6. Effects of genistein on the apoptosis of DLD-1 cells treated with increasing genistein concentrations. Apoptotic death was determined by enzyme-linked immunoabsorbent assay (ELIZA). For each concentration of genistein, mean values not sharing a common superscript (a, b, c) differ significantly ($P < 0.05$). All results represent the mean of four different experiments and are expressed as relative to the untreated control (relative death; data expressed as mean ± SD).

and 100 μM) caused a significant ($P < 0.01$) increase in DNA fragmentation also compared to 1 μM.

All results represent the mean of 4 different experiments, and the data obtained from the DNA fragmentation assay are expressed as relative to untreated control (relative death).

The apoptotic effect of genistein treatment was also evaluated by agarose gel electrophoresis. DNA fragmentation ladder was detectable in the cells treated with 30 μM of genistein, whereas no DNA fragmentation ladder was present at lower genistein concentrations (data not shown).
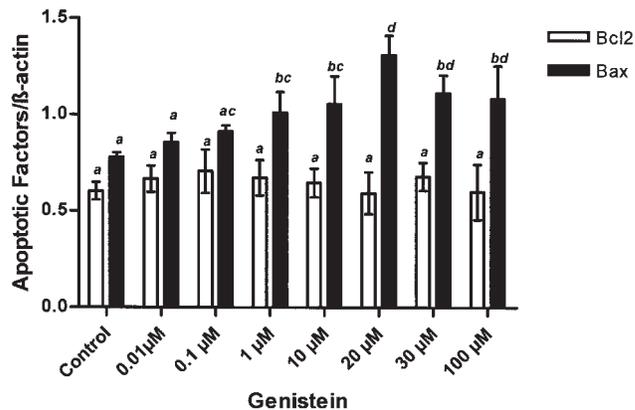

Figure 7. The effects of genistein on the Bax and Bcl-2 mRNAs expression in DLD-1 cells. For each concentration of genistein, mean values not sharing a common superscript (a, b, c) differ significantly ($P < 0.01$). All results represent the mean of four experiments and are expressed as Bax:β-actin ratio and Bcl-2:β-actin ratio, respectively (mean ± SD).

Effects of Genistein on Bax and Bcl-2 mRNA Expression

To examine the effect of genistein on the apoptotic pathway in human DLD-1 colon cancer cells, we evaluated mRNA expression of the apoptosis-related protein Bax and Bcl-2.

Both proapoptotic Bax and antiapoptotic Bcl-2 at mRNA level were expressed in untreated DLD-1 cells. In these experiments, the cells were treated with increasing genistein concentrations (from 0.01 μM to 100 μM), and the relative levels of Bax and Bcl-2 mRNA were evaluated by RT-PCR.

Cells treated with concentrations equal to or higher than 1 μM of genistein caused a significant ($P < 0.01$) increase in Bax mRNA expression compared to control cells and 0.01 μM. Besides, a peak in Bax mRNA expression was observed at 20 μM, which was also significantly higher ($P < 0.01$) than mRNA expression detected at genistein concentrations from 0.01 μM to 10 μM. On the contrary, Bcl-2 mRNA expression was not affected by genistein treatment (Figure 7).

Discussion

Genistein has been reported to possess a variety of biological activities that may account for its cancer-preventive effects (31), but it is not still clear whether consumption of soybean products is beneficial regarding the prevention of colon cancer.

On one hand, soybean products have been demonstrated to inhibit colon cell proliferation in a limited number of patients with colon polyps (32); on the other hand, in the animal models of experimental carcinogenesis, isoflavonoids have produced contrasting effects, probably due to different types of animals and models utilized (9,33–35).

In vitro experiments have provided more concordant data about the anticarcinogenetic properties of isoflavonoids, with particular reference to genistein. As a matter of fact, antiproliferative and proapoptotic activities of genistein have been proven in a wide range of cell lines including colon cancer cells (5,22). Nevertheless, the precise mechanisms through which isoflavonoids influence neoplastic cell growth are not completely known.

The main purpose of this study was to investigate the influence of genistein on the polyamine biosynthesis of DLD-1 cells. This can represent an interesting matter of study because it is known that high expression of ODC and increased polyamine concentrations are associated with fast proliferating cells (36,10) and that polyamine levels in cancer are significantly increased compared with normal and preneoplastic tissue (17).

Polyamines are able to stabilize chromatin and nuclear enzymes due to their ability to form complexes with organic polyanions such as groups of proteins and DNA. It was postulated that stabilization of the chromatin structure by polyamines may be a mechanism by which these molecules affect nuclear processes including cell division and apoptosis (11).

In this context, our main finding is that genistein induced significant decrease of ODC activity and polyamine levels in DLD-1 cells at concentrations starting from 1 μM. Particularly, DLD-1 cells showed a significant reduction in putrescine and spermidine contents, and in the spermidine:spermine ratio as well; spermine also decreased but not in a significant manner.

A similar polyamine response has also been observed in other cell lines, rodents, or humans treated with difluoromethyl ornithine, an ODC inhibitor (37), as well as in humans treated with probiotic bacteria (38). ODC is a key-regulator enzyme in the polyamine metabolism, being now considered as a true oncogene (39), and the marked decrease in its activity after genistein administration may account for the observed variations in the single polyamine contents. It is known that ODC activity influences mainly putrescine and spermidine levels, which are more involved in cellular proliferation than spermine; the latter is implicated essentially in the cellular differentiation and neoplastic transformation, with diverse processes involved in maintaining its critical levels (40). Nevertheless, the significant and inverse relationship that we found between genistein concentrations and the total polyamine levels suggests the influence of this isoflavone on the polyamine content in DLD-1 cells. The inhibitory effect exerted on the DLD-1 cell proliferation was further supported also by our data obtained with [^{3}H]–thymidine incorporation and the MTT test, although a significant inhibition for these 2 markers of proliferation occurred with genistein concentrations 10-fold higher. Probably, small amounts of genistein may be sufficient to produce inhibition in the biosynthesis of polyamines, which are necessary for cells to initiate their proliferative processes. As a matter of fact, the polyamine synthesis represents an early event during G1 phase of cell cycle (36).

Genistein produces effects suggestive for estrogenicity given that 1) it is structurally similar to estrogens, 2) it is capable of binding to the 2 ERs—ERα and ERβ subtype (13), 3) the ER complexes formed by estrogen and genistein are functionally equivalent (8), and 4) it can induce estrogen-responsive gene products (41).

Because DLD-1 express ERβ and genistein displays higher affinity with this type of receptor (13), it may be supposed that phytoestrogens, acting like estrogens do, could actively modulate proliferation also in cells from organs not considered classical sex steroid targets. Therefore, genistein modulation of neoplastic colon cell growth may occur at the transcriptional level consequential to an interaction between the genistein-receptor complex and some peculiar DNA sequences called hormone-responsive elements (42). This event can represent a regulatory step in the biosynthesis of specific molecules involved in cell proliferation such as polyamines.

To determine whether genistein induces apoptosis, we examined the Bcl-2 (apoptosis suppressor) and Bax (apoptosis inducer) mRNA levels and DNA fragmentation. Our data show that genistein administration starting from 10 μM exerted a significant apoptotic induction in DLD-1 cells as demonstrated by ELISA. Of note, 1 μM of genistein was adequate to increase significantly Bax mRNA whose expression precedes the DNA fragmentation. On the contrary, the Bcl-2 mRNA expression resulted to be unaffected by treatment. In addition, our data showed that for each genistein concentration, the Bax gene expression in DLD-1 cells was always higher than that of Bcl-2. This is an important evidence because it is well known that the decline of the Bcl-2:Bax ratio is crucial for the apoptosis induction (43).

These findings let us suppose that genistein effectively promotes the programmed cell death by upregulation of the Bax gene. This seems in agreement with data previously obtained in HT-29 human colon cancer cells in which the induction of apoptosis by the flavone was found to be associated also with altered transcript levels of genes involved in the apoptotic mechanisms (5).

We can presume that mechanisms by which genistein may affect growth of DLD-1 cells involve either an induction of apoptosis or a decrease in cell proliferation rate by involving a lessening of ODC activity and the polyamine levels. These properties may represent another antiproliferative mechanism offered by genistein in the protection against colon cancer.

As regards the different doses of genistein used in this study, concentrations from 0.01 to 10 μM may be considered as falling within or near the range of physiological levels in blood (21). Besides, it has been proven that according to the administered concentrations, genistein can exert different effects on cell proliferation in human intestinal cells. In fact, Chen et al. (24) demonstrated that in Caco-2 cells, genistein at a low dose (3.7 μmol/L) exerted a proliferative effect, whereas genistein concentrations ranging from 26 to 111 μmol/L inhibited intestinal cell proliferation. This effect was investigated by us on the polyamine metabolism in DLD-1

cells, and interestingly, this dual behavior in the proliferation of this cell line was not confirmed. In general, the minimum concentration of genistein required to affect proliferation in human colorectal tumor cell lines is not well known, and it varies according to the examined tumor cell line (44).

Over 300 plants and plant products contain phytoestrogens, and it has been hypothesized that they may have an effect in the modification of cancer risk by diet; thus, the possibility to regard genistein as a representative phytochemical functional food has made it attractive for in vivo studies on cancer risk. Our results obtained in vitro using a wide range of genistein concentrations, including values falling within the physiological blood levels in human, support indications for a diet rich in this isoflavone in terms of prevention for colorectal cancer. However, to obtain a therapeutic anticarcinogenetic effect from genistein, a supraphysiological intervention would be required. Probably, combinations of genistein with polyamine inhibitors and/or analogues as well as estrogens would enhance its properties, representing a suitable option for chemoprevention and/or treatment of colon cancer.

References

1. Messina MJ, Persky V, Setchell KDR, and Barnes, S: Soy intake and cancer risk: a review of the in vitro and in vivo data. *Nutr Cancer* **21,** 113–131, 1994.

2. Adlercreutz H, Goldin BR, Gorbach SL, Hockerstedt KAV, Watanabe S, et al.: Soybean phytoestrogen intake and cancer risk. *J Nutr* **125,** 757S–770S, 1995.

3. Adlercreutz H: Phyto-oestrogens and cancer. *Lancet Oncol* **3,** 364–373, 2002.

4. Onozawa M, Fukuda K, Ohtani M, Akaza H, Sugimura T, et al.: Effects of soybean isoflavones on cell growth and apoptosis of the human prostatic cancer cell line LNCaP. *Jpn J Clin Oncol* **28,** 360–363, 1998.

5. Wenzel U, Kuntz S, Brendel MD, Daniel H: Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res* **60,** 3823–3831, 2000.

6. Booth C, Hargreaves DF, Hadfield JA, McGown AT, and Potten CS: Isoflavones inhibit intestinal epithelial cell proliferation and induce apoptosis in vitro. *Br J Cancer* **80,** 1550–1557, 1999.

7. Watanabe T, Kondo K, and Oishi M: Induction of in vitro differentiation of mouse erythroleukemia cells by genistein, an inhibitor of tyrosine protein kinases. *Cancer Res* **51,** 764–768, 1991.

8. Knight DC, Eden JA: Phytoestrogens—a short review. *Maturitas* **22,** 167–175, 1995.

9. Hawrylewicz EJ, Zapata JJ, and Blair WH: Soy and experimental cancer: animal studies. *J Nutr* **125,** 698S–708S, 1995.

10. Tabor CW and Tabor H: Polyamines. *Annu Rev Biochem* **53,** 749–790, 1984.

11. Thomas T and Thomas TJ. Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell Mol Life Sci* **58,** 244–258, 2001.

12. Seiler N: Polyamine metabolism. *Digestion* **46** Suppl 2, 319–330, 1990.

13. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, et al.: Interaction of estrogenic chemical and phytoestrogens with estrogen receptor β. *Endocrinology* **139,** 4252–4263, 1998.

14. Sica V, Nola F, Contieri E, Bova R, Masucci MT, et al.: Estradiol and progesterone receptors in malignant gastrointestinal tumors. *Cancer Res* **44,** 4670–4674, 1984.

15. Linsalata M, Messa C, Russo F, Cavallini A, and Di Leo A: Estrogen receptors and polyamine levels in human gastric carcinoma. *Scand J Gaxstroenterol* **29,** 67–70, 1994.

16. Di Leo A, Messa C, Russo F, Misciagna G, Guerra V, et al.: Prognostic value of cytosolic estrogen receptors in human colorectal carcinoma and surrounding mucosa. Preliminary results. *Dig Dis Sci* **9,** 2038–2042, 1994.

17. Di Leo A, Linsalata M, Cavallini A, Messa C, and Russo F: Sex steroid hormone receptors, epidermal growth factor receptor and polyamines in human colorectal cancer. *Dis Colon Rectum* **35,** 305–309, 1992.

18. Messa C, Russo F, Pricci M, and Di Leo A: Epidermal growth factor and 17β-estradiol effects on proliferation of a human gastric cancer cell line (AGS). *Scand J Gastroenterol* **35,** 753–758, 2000.

19. Pricci M, Linsalata M, Russo F, Messa C, Amati L, et al.: Effects of 17β-estradiol administration on apoptosis and polyamine content in AGS cell line. *Anticancer Res* **21,** 3215–3220, 2001.

20. Messa C, Pricci M, Linsalata M, Russo F, and Di Leo A: Inhibitory effect of 17β-estradiol on growth and the polyamine metabolism of a human gastric carcinoma cell line (HGC-27). *Scand J Gastroenterol* **34,** 79–84, 1999.

21. Wang C and Kurzer MS. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. *Nutr Cancer* **28,** 236–247, 1997.

22. Arai N, Strom A, Rafter JJ, and Gustafsson J-A: Estrogen receptor β mRNA in colon cancer cells: growth effects of estrogen and genistein. *Biochem Biophys Res Commun* **270,** 425–431, 2000.

23. Kuntz S, Wenzel U, and Daniel H: Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. *Eur J Nutr* **38,** 133–142, 1999.

24. Chen A-C and Donovan SM: Genistein at a concentration present in soy infant formula inhibits Caco-2BBe cel proliferation by causing G2/M cell cycle arrest. *J Nutr* **134,** 1303–1308, 2004.

25. Cavallini A, Messa C, Pricci M, Caruso ML, Barone A, et al.: Distribution of estrogen receptor subtypes, expression of their variant forms, and clinicopathological characteristics of human colorectal cancer. *Dig Dis Sci* **47,** 2720–2728, 2002.

26. Linsalata M, Caruso MG, Leo S, Guerra V, D'Attoma B, et al.: Prognostic value of tissue polyamine levels in human colorectal carcinoma. *Anticancer Res* **22,** 2465–2470, 2002.

27. Garewal HS, Sloan D, Sampliner RE, and Fennerty B: Ornithine decarboxylase assay in human colorectal mucosa: methodological issues of importance to quality control. *Int J Cancer* **52,** 355–358, 1992.

28. Cifone MG, D'Alò S, Parroni R, Millimaggi D, Biordi L, *et al.*: Interleukin-2-activated rat natural killer cells express inducible nitric oxide synthase that contributes to cytotoxic function and interferon-γ production. *Blood* **93,** 3876–3884, 1999.

29. Lamon-Fava S, Ordovas JM, and Schaefer EJ: Estrogen increase Apolipoprotein (Apo) A-I secretion in Hep G2 cells by modulating transcription of the Apo A-I gene promoter. *Arterioscler Thromb Vasc Biol* **19,** 2960–2965, 1999.

30. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, and Vogt PH: Multiplex PCR: critical parameters and step-by-step protocol. *Biotechniques* **23,** 504–511, 1997.

31. Barnes S, Peterson TG, and Coward L: Rationale for the use of genistein-containing soy matrices in chemoprevention trials for breast and prostate cancer. *J Cell Biochem Suppl* **22,** 181–187, 1995.

32. Mobarhan S, Keshavarzian A, Frommel T, Chejfec G, Young S, et al.: The mechanism of chemopreventive effects of soy product isoflavones (IF) in human colonic polyps [Abstract]. *Gastroenterology* **114,** A645, 1998.

33. Helms JR and Gallaher DD: The effect of dietary soy protein isolate and genistein on the development of preneoplastic lesions (aberrant crypts) in rats. *J Nutr* **125,** 713S–716S, 1995.

34. Davis MJ, Bowey EA, Adlercreutz H, Rowland IR, and Rumsby PC: Effects of soy or rye supplementation of high-fat diets on colon tumor development in azoxymethane-treated rats. *Carcinogenesis* **20,** 927–931, 1999.

35. Rao CV, Wang CX, Simi B, Lubet R, Kelloff JG, et al.: Enhancement of experimental colon cancer by genistein. *Cancer Res* **57,** 3717–3722, 1997.

36. Pegg AE: Polyamine metabolism and its importance in neoplastic growth as a target for chemotherapy. *Cancer Res* **48,** 759–774, 1988.

37. Meyskens Jr FL and Gerner EW: Development of difluoro-methylornithine (DFMO) as a chemoprevention agent. *Clin Cancer Res* **5,** 845–851, 1999.

38. Linsalata M, Russo F, Berloco P, Caruso ML, Di Matteo G, et al.: The influence of lactobacillus brevis on ornithine decarboxylase activity and polyamine profiles in helicobacter pylori-infected gastric mucosa. *Helicobacter* **9,** 165–172, 2004.

39. Auvinen M, Paasinen A, Andersson LC, and Holtta E: Ornithine decarboxylase activity is critical for cell transformation. *Nature* **360,** 355–358, 1992.

40. Deloyer P, Peulen O, and Dandrifosse G: Dietary polyamines and non-neoplastic growth and disease. *Eur J Gastroenterol Hepatol* **3,** 1027–1032, 2001.

41. Joung KE, Kim YW, and Sheen YY: Assessment of the estrogenicity of isoflavonoids, using MCF-7-ERE-Luc cells. *Arch Pharm Res* **26,** 756–762, 2003.

42. Bolander FF: *Molecular endocrinology.* San Diego, CA: Academic, 1989, pp 205–227.

43. Pawlowsky J and Kraft AS: Bax-induced apoptotic cell death. *Proc Natl Acad Sci* **97,** 529–531, 2000.

44. Zhu Q, Meisinger J, Van Thiel DH, Zhong Y, and Mobarhan S: Effects of soybean extracts on morphology and survival of Caco-2, SW620, and HT-29 cells. *Nutr Cancer* **42,** 131–140, 2002.