RESEARCH ARTICLE

Soy isoflavone genistein induces cell death in breast cancer cells through mobilization of endogenous copper ions and generation of reactive oxygen species

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Received: July 15, 2010
Revised: October 1, 2010
Accepted: October 9, 2010

Scope: Worldwide geographical variation in cancer incidence indicates a correlation between dietary habits and cancer risk. Epidemiological studies have suggested that populations with high isoflavone intake through soy consumption have lower rates of breast, prostate, and colon cancer. Isoflavone genistein in soybean is considered a potent chemopreventive agent against cancer. Although several mechanisms have been proposed, a clear anticancer action mechanism of genistein is still not known.

Methods and results: Here, we show that the cytotoxic action of genistein against breast cancer cells involves mobilization of endogenous copper. Further, whereas the copper specific chelator neocuproine is able to inhibit the apoptotic potential of genistein, the molecules which specifically bind iron (desferroxamine mesylate) and zinc (histidine) are relatively ineffective in causing such inhibition. Also, genistein-induced apoptosis in these cells is inhibited by scavengers of reactive oxygen species (ROS) implicating ROS as effector elements leading to cell death.

Conclusions: As copper levels are known to be considerably elevated in almost all types of cancers, in this proof-of-concept study we show that genistein is able to target endogenous copper leading to prooxidant signaling and consequent cell death. We believe that such a mechanism explains the anticancer effect of genistein as also its preferential cytotoxicity towards cancer cells.

Keywords:
Apoptosis / Breast cancer / Copper / Genistein / Reactive oxygen species

1 Introduction

Cancer is a growing health problem around the world particularly with the steady rise in life expectancy. The concept that cancer can be prevented, or its onset postponed, by certain diet-derived substances, has in the recent years attracted considerable interest. It has been estimated that more than two-third of human cancers could be prevented through appropriate lifestyle modification including dietary habits. Plant polyphenols are important components of human diet and a number of them are considered to possess chemopreventive and therapeutic properties against cancer. Epidemiological studies have indicated that populations with high isoflavone intake through soy consumption have lower incidence of breast, prostate, and colon cancer [1]. Soybeans are a rich and relatively unique source of isoflavonoid genistein (4′,5,7-trihydroxyisoflavone), which is considered to be the major constituent responsible for the chemopreventive efficacy of soy. Anticancer effects of genistein have been demonstrated against cancer models, both *in vitro* and *in vivo* [2, 3].

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Abbreviation: ROS, reactive oxygen species

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Using a cellular system of peripheral lymphocytes isolated from human blood and alkaline single-cell gel electrophoresis (comet assay), we have earlier proposed a hypothesis that the prooxidant action of plant polyphenols, including genistein, may be an important mechanism for their anticancer- and apoptosis-inducing properties [4, 5]. Such a mechanism would involve mobilization of endogenous copper, possibly chromatin-bound copper, and the consequent prooxidant action. Over the past decade, we have proceeded to validate our hypothesis with considerable success [5–8]. We have also proposed that the preferential cytotoxicity toward cancer cells is explained by the elevated levels of copper in cancer tissues and cells [9]. Copper transporters are overexpressed in malignant cells, which aid the uptake and accumulation of excess of copper [10]. The reason for an increased copper concentration in tumors is not clearly understood. However, copper might be required for the expression of ceruloplasmin, the major copper-binding protein that is also elevated in cancer cells [11] and has been proposed to be an endogenous angiogenic stimulator [12]. As a further confirmation of our hypothesis, in this proof-of-concept study, we show that genistein-induced cytotoxicity against breast cancer cells involves mobilization of endogenous copper and the consequent prooxidant action.

2 Materials and methods

2.1 Materials

Genistein, metal chelators, and reactive oxygen species (ROS)-scavengers were purchased from Sigma (St. Louis, MO, USA). Breast cancer lines, MDA-MB-231, and MDA-MB-468 were obtained from ATCC (Manassas, VA, USA). Cells were cultured in a 5% CO_2-humidified atmosphere at 37°C. Stock solution of genistein was made in DMSO while stock solutions of metal chelators were in PBS. All stocks were always made fresh.

2.2 Cell growth inhibition studies by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

MTT assay was performed exactly as described earlier [13]. Briefly, MDA-MB-231 and MDA-MB-468 cells were seeded at a density of 2×10^3 cells *per* well in 96-well microtiter culture plates. After overnight incubation, cells were exposed to the indicated concentrations of genistein. Chelators, scavengers, or vehicle control were added in individual assays as mentioned in their respective experiments. Each treatment had eight replicate wells and the amount of DMSO in reaction mixture never exceeded 0.1%. Moreover, each experiment was repeated at least three times.

2.3 Histone/DNA ELISA for detection of genistein-induced apoptosis

The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in breast cancer cells treated with genistein, following the vendors protocol [13]. Chelators, scavengers, or vehicle control was added in individual assays as mentioned in their respective experiments.

2.4 Soft agar colonization assay

MDA-MB-231 cells (3×10^4) were plated in a 0.5 mL of culture medium containing 0.3% w/v top agar layered over a basal layer of 0.7% w/v agar (with complete culture medium) in 24-well plates. At the time of seeding, the culture was supplemented with genistein and chelators. After ~3 wk, colonies (>50 cells) were counted [13]. Experiments were carried out in quadruplicate, and the mean values are reported.

2.5 Spectrophotometric detection of Cu(II) reduction by genistein

The selective sequestering agent bathocuproine was employed to detect reduction of Cu(II) to Cu(I) by recording the formation of bathocuproine–Cu(I) complex, which absorbs maximally at 480 nm. The reaction mixture (3.0 mL) contained 3 mM Tris–HCl (pH 7.5), fixed concentration of Cu(II) (or Cu(I) for positive control) (100 μM), bathocuproine (300 μM), and genistein (50 μM). The reaction was started by adding Cu(II) and spectra were recorded immediately.

2.6 Statistical analysis

Results are expressed as mean ± SE of at least three independent observations. Student's *t*-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. p-values < 0.05 were considered statistically significant.

3 Results

3.1 Copper chelator neocuproine prevents genistein-induced cell proliferation inhibition in human breast cancer cells

The effect of genistein was observed on the proliferative potential of human breast cancer cells MDA-MB-231 and MDA-MB-468. Genistein caused a dose- and time-dependent inhibition of the proliferation of these cells, as assessed by the MTT assay (Fig. 1A). However, its inhibitory effect on

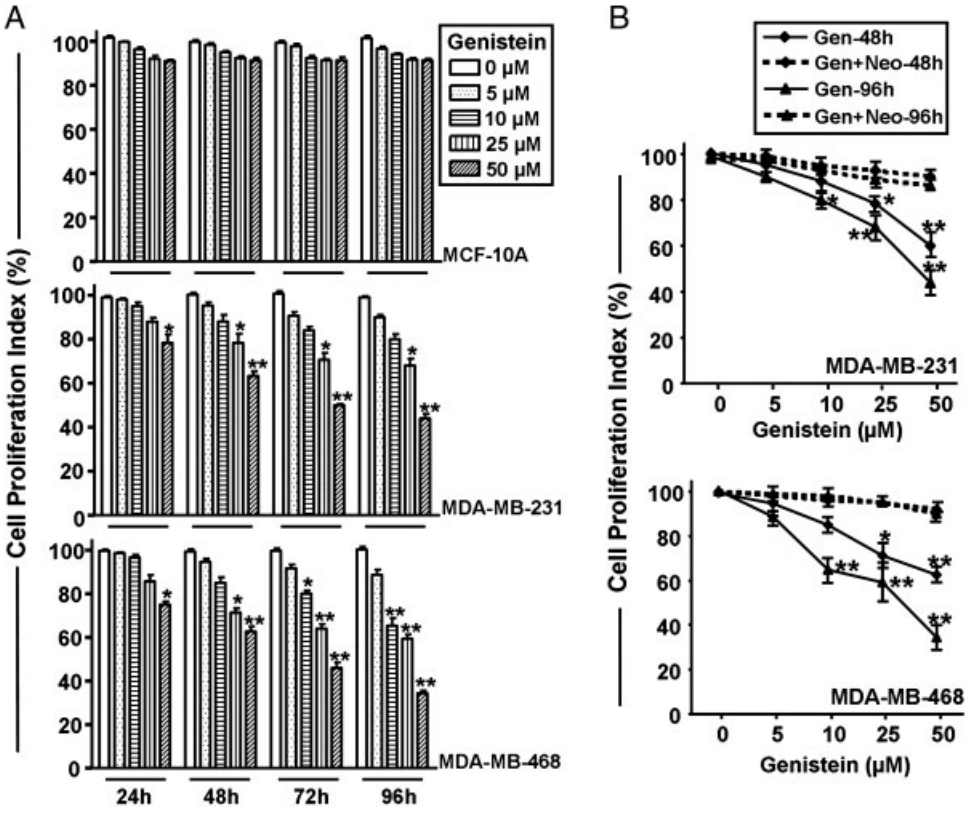

Figure 1. (A) Effect of genistein on cell growth of breast cancer cells and (B) protective effect of neocuproine, as detected by MTT assay. Cells were incubated with increasing concentrations of genistein (Gen), as indicated, in the presence or absence of 50 μM Neocuproine (Neo). All results are expressed as percentage of control (±SE). $^{*}p<0.05$ and $^{**}p<0.01$ compared to respective control (0 μM genistein).

the proliferation of 'normal' breast epithelial cells, MCF-10A, was found to be non-significant (Fig. 1A) thus verifying a cancer cell-specific anticancer effect. Further, when breast cancer cells MDA-MB-231 and MDA-MB-468 were treated with genistein in the presence of Cu(I)-specific chelator neocuproine, no such genistein-induced inhibition of cell proliferation was seen in both the cancer cell lines (Fig. 1B). These results suggest the involvement of endogenous copper, and Cu(I) as essential element, in the pathway that leads to cell growth inhibition by genistein.

3.2 Effect of redox metal chelators on genistein-induced apoptotic cell death

Next, we tested the apoptosis-inducing potential of genistein against MDA-MB-231 and MDA-MB-468 cells. The results obtained from Histone/ELISA assay (Fig. 2) clearly show that the exposure of breast cancer cells to 50 μM genistein led to time-dependent rise in absorbance at 405 nm. This indicates an increase in apoptosis induction as a consequence of enhanced internucleosomal fragmentation, a hallmark of apoptosis. In MCF-10A, genistein was not found to induce any apoptosis (results not shown) that again shows a cancer cell-specific activity of this anticancer agent. Furthermore, in the presence of neocuproine, genistein-induced apoptosis was significantly inhibited. On the contrary, iron-specific chelator desferioxamine mesylate and

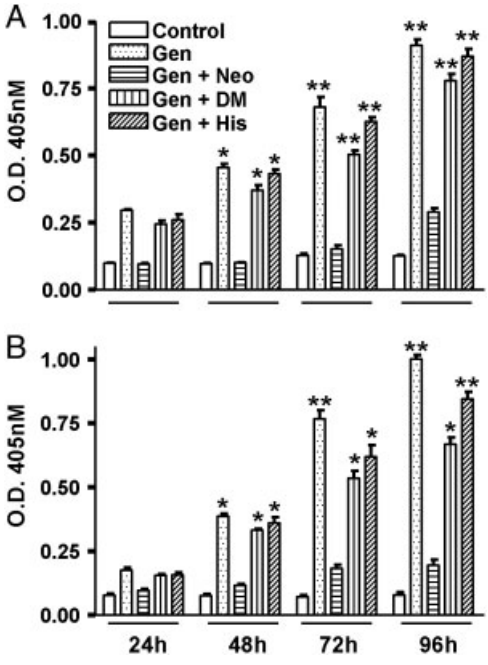

Figure 2. Effect of redox-metal-specific chelators on apoptosis induction by genistein in breast cancer cell lines (A) MDA-MB-231 and (B) MDA-MB-468. Gen, genistein (50 μM); Neo, neocuproine (50 μM); DM, desferoxamine mesylate (50 μM) and His, histidine (50 μM). $^{*}p<0.05$ and $^{**}p<0.01$ compared to respective vehicle control.

zinc-specific chelator histidine did not afford significant protection against genistein-induced cell death.

As a further proof, we looked at other markers of apoptosis, such as Bcl-2, Bax, and cleaved caspase-3 in MDA-MB-231 cells (Fig. 3). Genistein treatment resulted in down-regulation of antiapoptotic Bcl-2, upregulation of proapoptotic Bax and generation of active caspase-3, whereas neocuproine was able to almost completely reverse these genistein-induced effects, iron and zinc chelators were ineffective. These observations support the above results (Fig. 2) that copper chelator effectively abrogates the apoptosis-inducing effect of genistein in breast cancer cells.

3.3 Neocuproine circumvents genistein-induced suppression of clonogenic potential

Clonogenic or colony formation assay is an *in vitro* assay to assess a cell's ability to grow unattached to a surface indicating its metastatic potential [13]. As shown in Fig. 4, treatment of metastatic cell line MDA-MB-231 with two different doses of genistein resulted in the reduction of anchorage-independent colonies. Although iron and zinc chelator did not interfere with the ability of genistein to inhibit colony formation, copper chelator neocuproine nullified the effect of genistein and the number of anchorage-independent colonies was found similar to that of vehicle control.

3.4 Reduction of Cu(II) to Cu(I) by genistein

We have previously shown that polyphenols that oxidatively cleave DNA are able to reduce Cu(II) to Cu(I) [6]. Since the cytotoxic action of genistein against cancer cells was inhibited by copper chelator, the ability of genistein to reduce Cu(II) was examined. The production of Cu(I), formed as a result of reduction of Cu(II) by genistein was analyzed using bathocuproine, a Cu(I)-sequestering agent that binds specifically to the reduced form of copper, Cu(I), but not to the oxidized form, Cu(II). The Cu(I)-chelate exhibits an absorption maximum at 480 nm. As shown in Fig. 5, neither Cu(II) nor genistein interfered with the maxima, whereas genistein+Cu(II) reacted to generate Cu(I) which complexed with bathocuproine to generate a peak at 480 nm. The results show that genistein is able to reduce Cu(II) to Cu(I) contributing to its redox cycling.

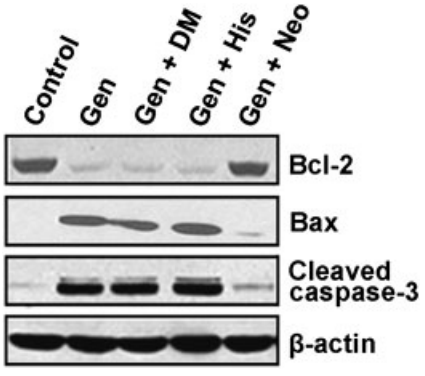

Figure 3. Effect of chelators on genistein-induced changes in markers of apoptosis in MDA-MB-231 cells after 96 h of treatment. Gen, genistein (50 μM); DM, desferoxamine mesylate (50 μM); His, histidine (50 μM) and Neo, neocuproine (50 μM). β-actin protein was used as protein loading control for the blot.

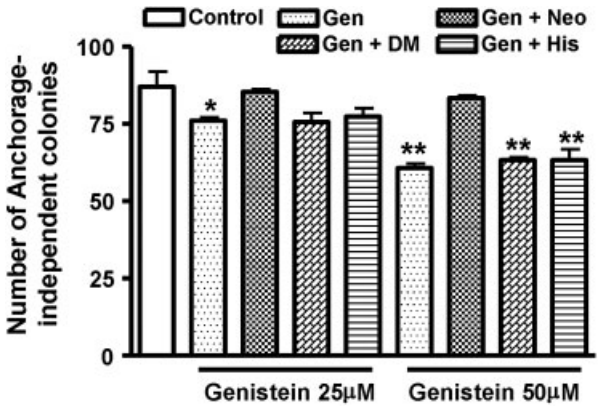

Figure 4. Effect of chelators on clonogenic potential of MDA-MB-231 cells treated with genistein (Gen). Neo, neocuproine (50 μM); DM, desferoxamine mesylate (50 μM) and His, histidine (50 μM). $^{*}p<0.05$ and $^{**}p<0.01$ compared to respective vehicle control.

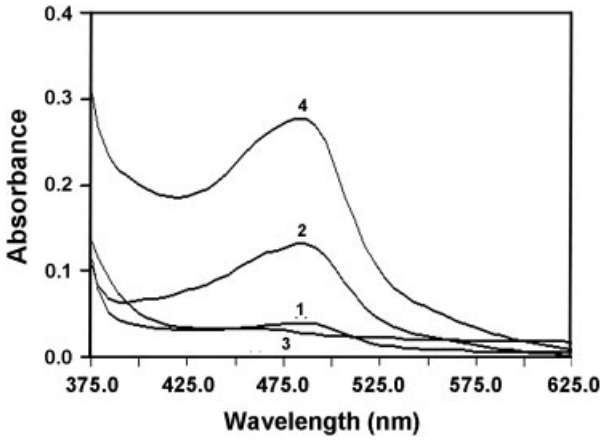

Figure 5. Detection of genistein-induced Cu(I) production. Trace 1, bathocuproine with 100 μM Cu(II); trace 2, bathocuproine with 100 μM Cu(I), trace 3,bathocuproine with 50 M genistein; trace 4, bathocuproine with 50 μM genistein and 100 μM Cu(II).

3.5 Involvement of reactive oxygen species in genistein-induced cell death pathway

Reoxidation of Cu(I) to Cu(II) in the presence of molecular oxygen leads to the generation of ROS [6]. In order to

Table 1. Effect of ROS scavengers on genistein activity in breast cancer cells

		Proliferation	% inhibition by genistein	Effect of scavengers[a)]	Apoptosis	Effect of scavengers[a)]
		Cell growth (%)			Fold increase[b)]	
MDA-MB-231	Untreated	100±3.88	–	–	–	–
	Gen	57.80±3.15*	42.20	–	4.96*	–
	+TU	80.20±1.88*	19.80	52.94	2.58*	47.86
	+SOD	73.90±1.46*	26.10	38.09	3.35*	32.40
	+Cat	64.20±2.37*	35.80	14.98	4.14*	16.34
MDA-MB-468	Untreated	100±3.43	–	–	–	–
	Gen	20.40±1.74*	79.60	–	9.83*	–
	+TU	63.50±0.98*	36.50	54.07	5.32*	45.87
	+SOD	48.95±1.80*	51.05	35.92	6.99*	28.93
	+Cat	36.00±1.31*	64.00	19.50	7.83*	20.37

Gen, 50 μM Genistein; TU, 700 μM Thiourea; SOD, 100 μg/mL superoxide dismutase; Cat, 100 μg/mL catalase.
a) Inhibition of genistein activity by scavengers (%).
b) Fold increase in apoptosis relative to untreated control. *$p<0.05$ compared to untreated control.

examine such a possibility, the effect of various scavengers of ROS such as superoxide dismutase, catalase, and thiourea was tested on genistein-induced cytotoxic action against breast cancer cells. Superoxide dismutase and catalase remove superoxide and H_2O_2, respectively, and thiourea removes hydroxyl radicals. As summarized in Table 1, all the three scavengers caused inhibition of genistein-induced antiproliferative and apoptotic activities in both the breast cancer cell lines. From the data we conclude that the genistein-induced cell death is mediated by the formation of ROS. Generation of superoxide anion may spontaneously lead to the formation of H_2O_2, which in turn causes the formation of hydroxyl radical through oxidation of reduced copper (Fenton reaction). Most cancer cells have an imbalance in antioxidant enzymes compared with normal cells [14]. In cancer cells, ROS levels can overwhelm the cells' antioxidant capacity, leading to irreversible damage and apoptosis [15].

4 Discussion

Plant-derived polyphenolic compounds including genistein have attracted considerable interest for their anticancer properties. However, the precise mechanism of their anticancer effects remains to be elucidated. Based on our own observations and those of others, we have proposed a mechanism of DNA fragmentation in cancer cells by plant polyphenolics that involves mobilization of intracellular copper [5]. Over the years we have validated the hypothesis: (i) an *in vitro* reaction between plant polyphenols, Cu(II) and DNA leading to DNA cleavage has been characterized [6, 16], (ii) we have shown that polyphenols are capable of mobilizing endogenous copper ions from cells leading to cellular DNA breakage [7, 8], (iii) we have also demonstrated that nuclear copper is mobilized in the above oxidative cellular DNA breakage [17]. Here, we show that polyphenol genistein-induced growth inhibition in breast cancer cell lines is inhibited by copper chelator to a significant extent whereas iron and zinc chelators are relatively ineffective.

This study identifies endogenous copper as a novel molecular target for cytotoxic action of genistein against cancer cells. A number of polyphenols, including genistein, have been shown to be cytotoxic to cancer cells leading to apoptotic cell death in various cell lines but not in normal cells [18, 19]. Such preferential cytotoxicity toward cancer cells is explained by the fact that serum [20], tissue [21], and intracellular [22] copper levels are significantly elevated in various malignancies. Since cancer cells contain elevated levels of copper, they may be more subject to electron transfer with polyphenols [6, 23] to generate ROS. It may be mentioned that although Fe^{3+} and Cu^{2+} are the most redox-active of the metal ions in living cells, several reports in the literature have shown that it is only the copper, which is significantly elevated in cancer patients [24, 25]. Interestingly, normal breast epithelial MCF10A cells have earlier been shown to possess no detectable copper [26] that might explain their resistance to genistein as observed here (Fig. 1A).

It is known [27] that the concentration of genistein is higher in individuals who consume a soy-containing diet and such population has lower incidence of cancers. Consuming 35 g of soybeans/day, the amount consumed by average Chinese, provides an intake of ~50 μg (185 μmol) of genistein. It has been found that the plasma level of genistein in people on a soy-rich diet is 1–5 μM after metabolism and excretion [28]. Further, genistein is only one of the polyphenols consumed as part of the diet. Since all polyphenols such as flavonoids, tannins, *etc.* are active as prooxidants [5], the cumulative concentration,

bioavailibility and effect of these polyphenols could be considerably greater than dietary genistein alone. It, however, needs to be mentioned that the half-life of various dietary polyphenols *in vivo* is very short, since these compounds are rapidly metabolized [29]. This possibly accounts for the relative inefficiency of plant polyphenols as anticancer agents compared to clinically used anticancer drugs. In this regard, identification of a definitive anticancer mechanism of plant polyphenols would contribute to establish them as potent lead compounds for the synthesis of novel anticancer drugs.

The authors acknowledge the UGC and CSIR, New Delhi.The authors alone are responsible for the content and writing of this paper.

The authors have declared no conflict of interest.

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