Genistein modulates neuroblastoma cell proliferation and differentiation through induction of apoptosis and regulation of tyrosine kinase activity and N-myc expression

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Genistein is a specific inhibitor of protein tyrosine kinase (PTK) and is considered as a therapeutic candidate for various cancers. In this paper we investigate the effects of genistein on cell proliferation and differentiation in neuroblastoma (NB) cell lines and its possible mechanism of action. Genistein substantially inhibited the growth of five (N2A, JC, SKNSH, MSN and Lan5) of the six tumor cell lines examined in a dose-dependent manner with an IC50 value of ~5 μg/ml. The exception was GC cells. N2A cells were treated with genistein for 6 days and exhibited morphological features of differentiation, as evidenced by the development of dendritic extensions. Terminal deoxynucleotidyl transferase (TDT) histochemical staining showed a significant elevation in darkly stained nuclei in genistein-treated N2A cells compared with controls, indicating the occurrence of apoptosis. Fluorescent quantitation of DNA fragments confirmed apoptosis in genistein-treated N2A cells. To further elucidate the possible mechanisms by which genistein modulates NB cell growth and differentiation we investigated the effect of genistein on the activities of PTK and mitogen-activated protein (MAP) kinase and N-myc proto-oncogene expression in N2A cells. The results showed that genistein down-regulated intrinsic PTK activity by ~33% and inhibited insulin-like growth factor (IGF)-stimulated PTK activity by 75%. The effect of genistein on the intrinsic activity of MAP kinase was insignificant. In addition, genistein significantly reduced N-myc expression in a dose-dependent fashion. Our study suggests that genistein arrests cell growth and induces NB cell differentiation by mediating apoptosis and modulating PTK activity and N-myc proto-oncogene expression.

Introduction

Epidemiological studies indicate that worldwide differences in human cancer incidence in various organs depends on lifestyle and dietary habits (1–3). Diets high in soybean products have been associated with a reduced risk of incidence of certain human cancers (4–6) and animal studies have shown that soybean diets inhibit radiation- and carcinogen-induced tumors of mammary glands and liver (7–11). In recent years widespread attention has focused on the possible role of soybean consumption in reducing cancer risk. Soybeans contain, in relatively high concentrations, several compounds with demonstrated anticancer activities in experimental oncology models (5,10,11). Members of one class of these compounds are the isoflavones genistein (5,7,4-trihydroxyisoflavone) and daidzein (7,4-dihydroxyisoflavone) (6). Genistein has several biological activities that might account for its anticancer activities, including inhibition of protein tyrosine kinase (PTK*), topoisomerase II (Topo II) and ribosomal S6 kinase (RS6K), induction of apoptosis and cell differentiation and scavenging of reactive oxygen species (10,11).

Genistein specifically inhibits the growth of ras-oncogene-transfected NIH 3T3 cells without affecting the growth of normal cells (12) and diminishes PDGF-induced c-fos and c-jun expression in CH310T1/2 fibroblasts (13). In addition, genistein inhibits Topo II and RS6K by stabilizing a cleavable Topo II–DNA complex and modulating mRNA translation in vitro (14), which may lead to protein-linked DNA strand breaks and cell growth suppression of several malignant cell lines (15–19). Genistein has been shown to induce differentiation of human melanoma cells (20), neuroblastoma cells (21) and leukemia cells (22). In addition, genistein potently inhibits production of certain cytokines (23) and eicosanoid biosynthesis (24), probably through inhibition of PTK. The wide anticancer properties of genistein have been further evidenced by its suppression of human gastrointestinal cancer cell growth (25) and inhibition of endothelial cell angiogenesis relevant to tumor metastasis (26). Uckun et al. (27) reported that the biotherapeutic potency of B cell precursor leukemia is tremendously increased by targeting genistein to CD19-associated tyrosine kinases.

Although increasing evidence suggests that genistein may be an excellent candidate as an anticancer agent, little information exists as to the effects and mechanisms of action of genistein on neuroblastoma (NB). NB is a common childhood malignant tumor of the autonomic nervous system arising from primitive ectodermal cells. Genetic alterations of certain genes are believed to be linked to development of this malignancy. Amplification of the N-myc oncogene in human NBs is associated with advanced tumors and rapid disease progression, suggesting that oncogene amplification is an important determinant of tumor behavior (21). The poor prognosis for the majority of children in the advanced stages of NB underlines the search for newer therapeutic strategies. The objectives of this study are to investigate whether genistein is able to arrest cell growth and induce differentiation of NB cells and elucidate the possible mechanisms of action of genistein.

Materials and methods

Chemicals

Genistein, dimethyl sulfoxide (DMSO) and Hoechst dye were obtained from Sigma Chemical Co. (St Louis, MO). Poly(Glu, Na-Tyr, 4:1) PGT and myelin

*Abbreviations: PTK, protein tyrosine kinase; Topo II, DNA topoisomerase II; RS6K, ribosomal S6 kinase; NB, neuroblastoma; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; TDT, terminal deoxynucleotidyl transferase; DMEM, Dulbecco’s modified Eagle’s medium; MAP kinase, mitogen-activated protein kinase; IGF, insulin-like growth factor.

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basic protein were purchased from Signal Transduction Laboratories (Ventre, CT). Tissue culture media, supplements and antibiotics were obtained from Gibco (Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories Inc. (Logan, Utah). The terminal deoxynucleotidyl transferase (TDT) enzyme kit was from Boehringer-Mannheim (Indianapolis, IN). The RNA isolation solution was purchased from Tel-Test Inc. (Friendswood, TX) and the N-myc gene cDNA probe was purchased from Oncogene Science (Uniondale, NY).

Cell culture
NB cell lines N2A, JC, SKNSH, Lan5, MSN and GC were gifts from Dr Yan Wang (Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL) and the information on optimal medium and culture conditions were given by the provider. NB cell lines N2A, JC and SKNSH were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% streptomycin/penicillin, 1% l-glutamine, 1% sodium pyruvate solution and 10% FBS. NB cell lines MSN and Lan5 were grown in RPMI 1640 supplemented with 1% streptomycin/penicillin, 1% l-glutamine, 1% MEM and 15% FBS. The GC cell line was grown in DMEM with 1% streptomycin/penicillin, 1% MEM and 1% l-glutamine and 7% horse serum (Gibco). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. The viability of the control culture was >95%.

Assays for cell proliferation and differentiation
NB cells (2×10³) were counted with a hemacytometer and plated in triplicate in 6-well plates and allowed to attach to the wells for 24 h. Genistein was dissolved in DMSO and added to cell cultures at concentrations of 0, 1, 2.5, 5, 10 and 20 µg/ml in the appropriate medium for 72 h. The final DMSO concentration was 0.05% in all cell cultures. At the end of the experiment cells were trypsinized and quantitated using a hemacytometer. Cell viability was determined by trypan blue exclusion. For cell differentiation assay cells were plated in 6-well plates and allowed to adhere for 24 h. Cells were treated with various concentrations of genistein for up to 9 days. Cells were photographed on days 3, 6 and 9 after genistein incubation. Differentiation was identified as cell spread and formation of dendritic extensions.

Analysis of apoptosis
Apoptosis was analyzed in this study by three different methods: gel electrophoresis, TDT histochemical staining and quantitation for DNA fragmentation using a spectrophotofluorometer. For DNA ladder assay cells were incubated with genistein for 48 h and genomic DNA isolated. Ten micrograms of isolated DNA from the control and genistein-treated samples were electrophoresed on a 1% agarose gel and visualized under a UV lamp for evidence of fragmented DNA (28). For TDT histochemical staining assay of apoptosis control and genistein-treated NB cells were plated onto glass slides in triplicate and stained with a TDT staining kit (29). Slides were photographed and 100 nuclei/slide were counted, with the results being expressed as the number of darkly stained nuclei per 100 cells. Quantitation of fragmented DNA in the culture medium was performed using a spectrophotofluorometer as described by Jarvis et al. (28).

Analyzes of PTK and mitogen-activated protein (MAP) kinase activities
Poly(Glu, Na-Tyr, 4:1) PGT was used as substrate for PTK activity. A reaction mixture containing 200 µg protein from the control or genistein-treated cell lysate, 20 mM HEPES, 10 mM MgCl₂, 5 mM MnCl₂, 0.05 mM sodium vanadate, pH 7.5, was pre-incubated for 15 min. PGT substrate and 50 ng insulin-like growth factor (IGF) were added. The reaction was initiated by adding 2 µCi [γ-³²P]ATP mixed with 40 µM cold ATP. The mixture was incubated at 25°C for 30 min. The reaction was terminated by plating an aliquot of the reaction mixture onto Whatman filter paper. Filters were washed three times with 5% trichloroacetic acid, 1% disodium pyrophosphate and ether:methanol (50:50). Filters were dried and radioactivity counted in a scintillation counter. Results are expressed as pmol [³²P]-phosphorylated protein/mg protein. The assay for MAP kinase activity was similar to the method of PTK assay except for the use of myelin basic protein as substrate.

Northern blot analysis of N-myc expression
N2A cells were treated with genistein for appropriate times. Total RNA was isolated by RNA isolation solution and 20 µg RNA were loaded onto a 1.0% agarose gel containing 2.2 M formaldehyde and electrophoresed in 1× MOPS/EDTA buffer for ~3 h (70–80 V). After electrophoresis the gel was soaked in 0.1 M NaCl and 50 mM NaOH for 30 min, then in 0.1 M Tris–HCl, pH 7.5, for 30 min and finally in 2× SSC for 30 min. RNA in the agarose gel was transferred to a nitrocellulose membrane. After completion of transfer the nitrocellulose membrane was baked at 80°C for 2 h, placed in a sealable bag containing 15–20 ml pre-hybridization solution (50% formaformamide, 5× Denhardt’s solution, 5× SSC, 1% SDS and 100 µg/ml salmon sperm DNA) and incubated at 42°C for 4 h. cDNA probes of N-myc and β-actin were prepared using nick translation. β-Actin was used to quantitate the amount of RNA sample loaded in the gel electrophoresis. The labeled probes were added to the hybridization bag and incubated at 42°C overnight. Filters were washed and autoradiographs of the Northern blots were visualized by exposing the filter to X-ray film at ~70°C. The bands of N-myc and β-actin mRNA were quantitated using densitometry.

Statistical analysis
All experiments were repeated at least twice depending on reproducibility. Results are expressed as mean ± SE and all data were analyzed by ANOVA or Student’s t-test.

Results
Effect of genistein on NB cell growth
Six NB cell lines, N2A, SKNSH, JC, GC, MSN and Lan5, were used to examine the effect of genistein on cell growth. As shown in Figure 1, genistein substantially inhibited growth of five (N2A, JC, SKNSH, MSN and Lan5) of the six tested NB cell lines in a dose-dependent manner with an IC₅₀ value of ~5 µg/ml. GC cells were resistant to genistein treatment and the cell growth inhibition curve plateaued at concentrations >5 µg/ml genistein. Resistance of the GC cell line to genistein continued up to the cytotoxic concentration (data not shown).

Effect of genistein on differentiation of N2A cells
Genistein has been shown to induce differentiation of certain tumor cell lines (20–22). We further evaluated the effects of genistein on NB cell differentiation and observed that among the tested cell lines genistein is a potent inducer of terminal differentiation of cell line N2A in a dose-dependent fashion.
Fig. 2. Cell differentiation of N2A NB cells by genistein. N2A cells were treated with genistein at concentrations of 0 (A), 5 (B) or 20 µg/ml (C) for up to 6 days. Cells were photographed at days 2, 4 and 6 to monitor differentiation. Two experiments were performed in triplicate and the results show consistency. The photographs shown here were taken 6 days post-genistein treatment. Note the development of dendritic extensions in (B) and (C).

(Figure 2). Cells growing in genistein-free medium are primarily round in shape. After treatment with 5 or 20 µg/ml genistein N2A cells became flattened and spread. At 20 µg/ml genistein cells were completely spread and developed extensive dendritic extensions, which are morphologically indicative of cell differentiation.

**Induction of apoptosis in N2A cells by genistein**

We also investigated whether genistein induced apoptosis of NB cells. A DNA ladder assay by gel electrophoresis did not reveal an apoptotic pattern in N2A cells treated with genistein for up to 6 days (Figure 3). Since DNA ladder assay lacks the sensitivity to detect DNA fragmentation in solid tumor cells (28), TDT staining was used to detect apoptosis of cells at earlier stages (29). A substantial elevation of darkly stained nuclei was observed in N2A cells treated with genistein for 24 h. Table I shows that TDT stained cells were significantly increased with 31% TDT-positive nuclei in genistein-treated cells versus 3% in controls ($P < 0.01$). To further confirm the apoptotic effect of genistein on NB cells a fluorometric method was used to quantitate fragmented DNA released into the medium by NB cells (28). Treatment of N2A cells with 1, 10 and 50 µg/ml genistein for 6 h led to a dose-dependent increase in DNA fragmentation (Table II). A statistically significant difference in DNA fragmentation was observed between the control and (10 or 50 µg/ml) genistein-treated cells ($P < 0.05$). Thus at least two assays showed that genistein significantly induces apoptosis in a NB cell line.
Table II. Quantitation of fragmented DNA in genistein-treated NB cells

<table>
<thead>
<tr>
<th>Genistein (µg/ml)</th>
<th>DNA fragmentation (fluorescence ratio)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>1</td>
<td>0.77 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>0.91 ± 0.04a</td>
</tr>
<tr>
<td>20</td>
<td>0.97 ± 0.01a</td>
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N2A cells were incubated with genistein for 6 h and analyzed for DNA fragmentation. DNA fragmentation is expressed as the ratio of DNA in the supernatant to total DNA (supernatant + pellet). Data are means ± SE from two independent experiments with each assay performed in duplicate. *P < 0.05 versus DMSO-treated controls.

![Fig. 4. Effect of genistein on PTK and MAP kinase activities.](image)

**Effect of genistein on intrinsic and IGF-stimulated PTK and MAP kinase activities**

PTK activity was determined as a function of phosphorylation of the substrate poly(Glu, Na-Tyr;4:1). Genistein down-regulated intrinsic PTK activity in a dose-dependent manner with ~33% reduction at 20 µM genistein (Figure 4A). In addition, genistein substantially inhibited IGF-stimulated PTK activity in N2A cells by ~75% (Figure 4B). A statistically significant difference in PTK activity was observed between mock control, IGF-stimulated and IGF/genistein-treated cells. Myelin basic protein was used as substrate to investigate the effect of genistein on MAP kinase activity in N2A cells. The results showed that genistein had no effect on intrinsic MAP kinase activity of NB cells at the selected concentrations when compared with untreated controls (Figure 4A and B). These experiments indicate that genistein down-regulates both intrinsic and mitogen-activated PTK activity but has little effect on intrinsic MAP kinase activity.

**Suppression of N-myc expression by genistein**

The proto-oncogene N-myc is known to be overexpressed in a number of NB cells. Amplification of the N-myc oncogene is frequently used to evaluate clinical prognosis of NB. Analyses of N-myc mRNA in untreated NB cell lines N2A, Lan5, MSN, JC, GC and SKNSH showed that all tested NB cells expressed N-myc to different extents (Figure 5). N2A cells were chosen for the genistein study because this cell line expresses significant amounts of N-myc and was used in the differentiation and apoptosis study. Figure 6A shows that N2A cells incubated with 5 or 20 µg/ml genistein for 1 h exhibited a dose-dependent decrease in N-myc mRNA expression with the housekeeping gene β-actin as internal standard. Densitometric quantitation indicated that genistein treatment significantly decreased N-myc expression in N2A cells by ~50% (Figure 6B). This experiment suggests that genistein is able to down-regulate N-myc overexpression in cell line N2A.

**Discussion**

It is well known that soybean products in the diet reduce the risk of cancer (4–6). A number of studies have shown that genistein inhibits tumor cell growth, suppresses oncogene expression and induces cell differentiation of malignant cells (9–11). Therefore, we have examined the biological effect of genistein on NB cell growth and differentiation. In the present study we have shown that genistein inhibits cell proliferation of NB cell lines N2A, SKNSH, MSN, Lan5 and JC in a dose-dependent manner. The IC50 value was ~5 µg/ml, which is consistent with those of genistein inhibition of other tumor cell lines reported by Barnes and others (10–17). In this study the GC cells appeared to be less responsive to genistein. There could be several reasons for this phenomenon: (i) the intrinsic malignancy of GC cells may be genetically determined differently from other NB cell lines; (ii) a confounding effect of the medium. Since GC cell have special medium requirements,
the difference in medium components may confer resistance to genistein. Several factors may play a role in the selectivity of genistein with respect to cell growth inhibition. Genistein potently inhibits activities of PTK (30,31), Topo II (32) and RSK6K (33). Several retroviral genes, such as src, yes, fgr, abl, fps, fes and ros, encode tyrosine-specific protein kinases, which are also associated with cellular receptors, e.g. EGF and PDGF receptors (34). This suggests that tyrosine phosphorylation plays an important role in cell proliferation and cell transformation. Genistein is a known inhibitor of PTK and may act as a potential anticancer agent.

NB is a peculiar tumor in its behavior as it can occasionally undergo spontaneous or chemically induced maturation or regression in vivo. One mechanism of tumor regression probably involves maturation of neoplastic cells into terminally differentiated, non-proliferating ganglion-like cells. A second mechanism closely associated with differentiation could be induction of programmed cell death (35). In this process most malignant cells die via an apoptotic mechanism, whereas cells with differentiation potential will survive as a result of selection. Our study indicates that genistein induces differentiation of N2A cells in a dose-dependent manner, as evidenced by development of neuritic extensions. An important fact is that genistein induces differentiation not only at the high concentration of 20 \( \mu g/ml \) but also at the cytostatic concentration of 5 \( \mu g/ml \).

McCabe and Orrenius (36) reported that genistein treatment of HL-60 and human thymocyte cells induced apoptosis. We assume that genistein may arrest NB cell growth by inducing apoptosis. However, a DNA ladder assay in this study did not show the characteristic DNA fragmentation pattern in genistein-treated NB cells. Despite overwhelming evidence in favor of internucleosomal DNA cleavage activity visualized by gel electrophoresis as a characteristic hallmark of apoptosis, several accounts of apoptosis have been reported that do not show a ladder pattern by this method, particularly in solid tumor cells. Endonuclease is known to play an important role in DNA cleavage during apoptosis, in particular at an early stage. Activation of endonuclease cleavage can be detected using TDT histochemical staining of apoptotic nuclei. We demonstrated that genistein-treated NB cells displayed a much higher number of darkly stained (TDT-positive) nuclei compared with untreated controls. The apoptotic effect of genistein was further confirmed by a fluorescent quantitation of fragmented DNA released by apoptotic cells, indicating that genistein is able to induce apoptosis or programed cell death in NB cells.

The apoptotic process plays a critical role in the development of the nervous system, where a large number of newly generated neuronal cells die by apoptosis. This ensures that the appropriate number of mature neurons is selected and eventually survives (37). The attempt to restore the normal rate of apoptosis in neoplastic tissues may represent a novel antitumor strategy. Apoptosis, known to be induced by a variety of stimuli, such as anticancer agents, differs from necrosis in many respects. The main difference is the active participation of cells in this process. During apoptosis loss of membrane integrity is typically preceded by chromatin condensation and internucleosomal cleavage of genomic DNA. Some malignant tumor cells express various components of the apoptotic program and may be susceptible to various pharmacological triggers which activate the cell death pathway.

The precise mechanism by which genistein modulates cell proliferation and differentiation of NB cell lines remains unclear. We postulate several possible mechanisms as to how genistein exerts its inhibitory action on NB cell growth. Genistein has been shown to be an inhibitor of several cytoplasmic tyrosine kinases, such as src and p210bcr-abl, which implies that genistein inhibits tyrosine phosphorylation events distal to membrane-bound growth factor receptors (30,31). The growth of cancer cells is largely dependent on the action of one or more oncogenes. Many of these oncogenes were found to express high or unregulated PTK activity and high levels of tyrosine phosphorylation have been associated with cancer cell growth in vivo (34). Thus genistein probably inhibits cell growth through blocking the action of PTK-associated oncogenes. We have demonstrated that genistein suppresses both 12-O-tetradecanoylphorbol-13-acetate- and UVB-induced proto-oncogenes c-fos and c-jun expression in mouse epidermis (38,39). Activated forms of oncogenes are known to be involved in cell growth control and transformation of normal cells via continuous or inappropriate activation of normal signal transduction pathways (34).

Protein phosphorylation and dephosphorylation are key events in the pathways that regulate cell growth and differentiation. Many transforming oncogenes and growth factor receptors contain PTK activity. This activity is thought to be a

Fig. 6. Down-regulation of N-myc expression in N2A NB cells by genistein. Total RNA (40 \( \mu g \)) from N2A cells treated with vehicle and genistein were loaded onto agarose/formaldehyde gels and N-myc mRNA was analyzed by Northern hybridization as described in Materials and methods. (A) Lane 1, negative control; lane 2, 5 \( \mu g/ml \) genistein; lane 3, 20 \( \mu g/ml \) genistein. (Upper) Hybridization with N-myc probe; (lower) hybridization with \( \beta \)-actin probe. (B) Densitometric quantitation of normalized ratio of N-myc/\( \beta \)-actin from three independent experiments with standard errors. \( *P < 0.05 \) and \( **P < 0.01 \) when compared with untreated NB cells.
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determining factor in the etiology of cancer (34). In assays with purified kinases genistein acts as a specific tyrosine kinase inhibitor, but has little or no effect on serine/threonine kinases, such as protein kinase C and cAMP-dependent protein kinase (30). Our studies show that genistein substantially down-regulates both intrinsic and mitogen-induced PTK activity in the N2A cell line. These results indicate that a decrease in protein tyrosine phosphorylation by genistein may be closely associated with inhibition of cell proliferation or induction of cell differentiation of NB cells.

The signal transduction pathways of mitogenic stimuli in NB cells are not clearly understood. It has been suggested that MAP kinase, which can be activated by phorbol esters and various growth factors, functions as an integration point for different pathways (40). X-Ray irradiation, phorbol esters and H2O2 have been shown to stimulate MAP kinase activity, probably through formation of reactive oxygen species in several studies (40,41). Our studies revealed that genistein had little effect on intrinsic MAP kinase activity and only exhibited mild inhibition of IGF-stimulated MAP kinase activity. Thus it appears that the anti-proliferative action of genistein is independent of MAP kinase in cell line N2A.

N-myc proto-oncogene is overexpressed in most NB cells and the degree of N-myc expression in NB tissues correlates well with differentiation grade and clinical prognosis (42). In the present study we have demonstrated that genistein dose-dependently reduced expression of N-myc proto-oncogene mRNA in NB cell line N2A, which was closely associated with genistein-induced differentiation of N2A cells. It is assumed that regulation of N-myc expression may be attributed to modulation of PTK activity. Our current knowledge of the biochemical and biological actions of genistein in prevention of cancer is incomplete. To date no correlation between genistein and N-myc expression in NB cells has been reported. The fact that genistein down-regulates N-myc and induces differentiation of NB cell lines may suggest the potential use of genistein in the therapy of NB in the future.

In summary, we have demonstrated that genistein arrests cell growth of NB cells except for cell line GC. At appropriate concentrations genistein induces programmed cell death and differentiation. Mechanistic studies show that genistein substantially down-regulates intrinsic and mitogen-stimulated PTK activity and N-myc expression, with little effect on MAP kinase. However, the fact that genistein substantially inhibits cell growth and induces cell differentiation of NB cells suggests that genistein may serve as a potential agent in the prevention or therapy of NB.

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