Induction of Differentiation and DNA Strand Breakage in Human HL-60 and K-562 Leukemia Cells by Genistein

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ABSTRACT

Genistein, an in vitro inhibitor of topoisomerase II and tyrosine kinases, suppressed growth and induced differentiation in HL-205 cells, a clonal population of the human promyelocytic HL-60 leukemia cells, and in K-562 cells, a clonal population of the human erythroid K-562 leukemia cells. Maturing HL-205 cells acquired either granulocytic or monocytic markers, namely, reactivity with the murine OKM1 monoclonal antibody, expression of nitroblue tetrazolium dye reduction, and staining for nonspecific esterase. The maturing K-562-J cells stained with benzidine, which indicates the presence of hemoglobin, an erythroid maturation marker. Although the acquisition of the maturation markers in both HL-205 and K-562-J cells was time dependent up to 6 days, the kinetics of this induction differed between the two cell types. Despite the in vitro inhibitory effect of genistein, treatment of either HL-205 or K-562-J cells with 150 μg/ml genistein for up to 16 h did not alter topoisomerase II activity (as determined by the unknotted assay) in their nuclear extracts. Analysis with the anti-photophosphoryse PY-20 murine monoclonal antibody indicated that treatment of K-562-J cells with genistein decreased the reactivity of the antibody with two of the cellular proteins. However, no reactivity with the PY-20 antibody was detected in untreated or genistein-treated HL-205 cells. An early event in the HL-205 and K-562-J cells, occurring after 1 h of treatment with 30–200 μg/ml genistein, was the induction of DNA damage as measured by an alkaline elution assay. This damage may be a contributing factor in the genistein-induced cell differentiation in the HL-205 and K-562-J cells.

INTRODUCTION

Decreased activity of either topoisomerases or tyrosine kinases has been implicated in the differentiation of a number of cell types (1–8). With regard to the inhibition of topoisomerases, the activity of Topo II is decreased when mouse erythroleukemia cells acquire a mature phenotype (5) and when HL-60 cells differentiate following treatment with novobiocin, a Topo II inhibitor, or phorbol 12-myristate 13-acetate, a phorbol diester (7, 8). With respect to the inhibition of tyrosine kinase, a reduction in the amounts of protein phosphotyrosine residues has been reported during granulocytic and monocytic maturation of the human promyelocytic HL-60 leukemia cells (1) and a reduction in the level of tyrosine-phosphorylated proteins has been observed in the erythroid differentiation of K-562 leukemia cells (2).

These observations raised the possibility that genistein, because of its dual action as a topoisomerase inhibitor and a tyrosine kinase inhibitor (9, 10), may induce cell differentiation.

To test this possibility, we investigated the ability of genistein to inhibit the activities of topoisomerase II and tyrosine kinases and to induce differentiation in the human HL-60 and K-562 leukemia cells. We report here that genistein induces a mature phenotype in both cell types and that this induction is associated with its effect on DNA strand breakage, possibly mediated by Topo II.

MATERIALS AND METHODS

Chemicals and Reagents. Genistein (4',5,7-trihydroxyisoflavone) and the murine PY-20 monoclonal antibody were purchased from ICN Biomedicals; novobiocin, proteinase K, TLCK from Sigma Chemical Co.; and polycarbonate membrane filters from Nucleopore Corp. The murine OKM1 monoclonal antibody was from Pharmingen Biological Corp. Knotted bacteriophage P4 DNA was isolated from the tailless capsids of the bacteriophages ∆viri I del(11). Topo II was purified from HL-625 as described by Drake et al. (12). Calf thymus Topo I was purchased from Bethesda Research Laboratories. Stock solutions of genistein and TLCK were dissolved in dimethyl sulfoxide at 30 mg/ml and stored at −70°C. Novobiocin was dissolved in double-distilled water at 10 mg/ml and stored at −20°C. [methyl-3H]Thymidine was purchased from ICN Radiochemicals. The chemicals were added to the culture once at the beginning of the treatment.

Cells. Culture Conditions, and Differentiation Markers. The HL-205 cell clone was isolated from HL-60 cells as described (13). The K-562-J cell clone was isolated in this laboratory by Dr. S. Maeda from the K-562 cells originally established from a chronic myelogenous leukemia patient (14). Cells were cultured in 100- or 150-mm tissue culture dishes at 1.5 × 10⁵ cells/ml of RPMI 1640 supplemented with 15% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), and cultured at 37°C in a humidified atmosphere of 8% CO₂ in air. Immunofluorescence tests for reactivity with the antibodies, staining for NSE activity, and the NBT dye reduction assay were all performed as previously described (15, 16). The presence of hemoglobin in the differentiating K-562-J cells was determined by the benzidine-staining assay (17).

Unknotted Assay for the Determination of Topo II Activity. The enzyme source was either Topo II purified from HL-625 cells (13) or serial dilutions of nuclear extracts of either untreated cells or cells treated with genistein. The preparation of the nuclear extracts, the P4 DNA substrate, and the electrophoresis conditions were previously described (8). Reaction mixtures of 20 μl contained 2 μl of enzyme preparation-50 mM Tris-Cl, pH 8.0-100 mM KCl-10 mM MgCl₂-0.5 mM dithiothreitol-0.5 mM EDTA-30 μg/ml bovine serum albumin-1 mM ATP. One unit of unknotted activity was defined as the amount of the enzyme that converts one-half of the knotted DNA to unknotted DNA during a 30-min incubation at 37°C as previously described (8).

Relaxation Assay for the Determination of Topo I Activity. Reaction mixtures of 20 μl contained 50 mM Tris-Cl, pH 7.4-50 mM KCl-10 mM MgCl₂-1 mM dithiothreitol-0.1 mM EDTA-30 μg/ml bovine serum albumin. The enzyme source was either purified Topo I (from calf thymus) or serial dilutions of nuclear extracts of either untreated cells or cells treated with genistein; the substrate was pUC8 DNA (90% supercoiled). The electrophoresis conditions were as described previously (8). One unit of relaxation activity was defined as the amount of the enzyme that converts one-half of the supercoiled DNA to relaxed DNA during a 30-min incubation at 37°C as previously described (8).

Topo II-mediated DNA Cleavage Assay. The conversion of supercoiled pUC8 DNA to its linear form was measured in the same reaction mixture as was used for the relaxation assay except that ATP was...
observed in the K-562-J cells, but the degree of decrease was less pronounced than in HL-205 cells (Figs. 1 and 2).

During a period of 6 days in culture, less than 7% of untreated HL-205 cells reacted with the murine OKM1 monoclonal antibody that detects a cell surface antigen common to mature myeloid cells (21) and less than 10% of the cells stained positively for NSE activity, a characteristic of monocytes/macrophages (22), or for NBT dye reduction, a marker of granulocytic maturation in these cells (16). Treatment of these cells with genistein resulted in not only a decrease in cell number but also an induction of cell differentiation (Fig. 3). In the HL-205 cells, genistein at 10 μg/ml caused a time-dependent increase in the percentage of cells reacting with the OKM1 antibody, staining for NSE activity, and exhibiting NBT dye reduction (Fig. 3). After 4 days of treatment, more than 95% of the cells reacted with the OKM1 antibody, about 50% of the cells exhibited NBT dye reduction, and 20% of the cells stained positively for NSE activity (Fig. 3). Under these conditions, less than 8% of the cells stained with trypan blue, a characteristic of dead cells. At a higher dose of genistein (20 μg/ml), the percentage of HL-205 cells staining for NSE activity rose to about 50% (Fig. 3).

Similarly, treatment of the K-562-J cells with genistein resulted in cell differentiation. At 10 μg/ml, the genistein caused a time-dependent increase in the percentage of K-562-J cells staining positively with benzidine (Fig. 4), which indicates the presence of hemoglobin, an erythroid maturation marker in these cells (17). On the seventh day, more than 50% of cells stained positively with benzidine, while during the 7 days of culture less than 5% of control cells stained positively with the dye (Fig. 4). Under these conditions, less than 5% of the cells stained with trypan blue. The increase in benzidine-stained cells was also dose dependent up to 10 μg/ml of genistein (Fig. 4). Higher doses of the agent were less effective. Genistein also did

Fig. 1. Effect of 10 μg/ml of genistein on the growth of HL-205 and K-562-J cells as a function of time. ○, untreated HL-205 cells; △, untreated K-562-J cells; ○, treated HL-205 cells; △, treated K-562-J cells.

Fig. 2. Effect of different concentrations of genistein on the growth of HL-205 (○) and K-562-J (△) cells after 4 days of treatment.

RESULTS

Induction of Differentiation in HL-205 and K-562-J Cells by Genistein. Genistein, an isoflavone compound isolated from the fermentation broth of Pseudomonas sp., is an in vitro inhibitor of tyrosinase, and tyrosine kinases (9, 10). This compound was tested for its ability to inhibit cell growth and to induce differentiation in the HL-205 and K-562-J cells. Treatment of HL-205 cells for up to 6 days with different concentrations of genistein caused a time-dependent (Fig. 1) and dose-dependent (Fig. 2) decrease in cell numbers. A similar effect was also
Fig. 3. Induction of differentiation of HL-205 cells treated with genistein. 
Left, time response; cells were treated with 10 μg/ml of genistein and the 
percentage of cells reacting with the OKM1 monoclonal antibody (●), reducing 
the NBT dye (●), and expressing NSE activity (▲) was determined. During the 6 
days in culture, less than 7% of the control cells reacted with OKM1 antibody, 
and less than 5% of the control cells reduced the NBT dye or expressed NSE 
activity. Right, dose response; cells were treated with increasing concentrations 
of genistein and the percentage of cells reacting with the OKM1 monoclonal 
antibody (●) and expressing NSE activity (▲) was determined after 4 days of 
treatment. Each point represents the mean ± SE of three independent determinations.

Fig. 4. Induction of differentiation of K-562 cells with genistein. Left, time 
response; cells were treated with 10 μg/ml of genistein and the percentage of 
benzidine-stained positive cells was determined as a function of time. During the 
7 days in culture, less than 5% of the control cells stained with benzidine. Right, 
dose response; cells were treated with increasing concentrations of genistein and the 
percentage of benzidine positive cells was determined after 7 days of treatment. 
Each point represents the mean ± SE of three independent determinations.

not cause an increase in the percentage of K-562-J cells reacting 
with the OKM1 antibody (data not shown).

These results indicate that genistein induces growth inhibition 
and differentiation in both the HL-205 and the K-562-J 
cells. The kinetics of this induction, however, differ between 
the two cell types.

Inhibition of Topoisomerase Activity and Stabilization of a 
Topo II-DNA Complex by Genistein. To confirm and extend 
previous studies indicating that genistein can inhibit the activities 
of DNA topoisomerases and tyrosine kinases (9, 10), we 
measured the effect of genistein on these activities using purified 
Topo I and Topo II and a cell preparation that contains the 
EGFTK. For comparison we included novobiocin, a known 
Topo I inhibitor, and TLCK, a known tyrosine kinase inhibi-
tor. The activity of Topo II was determined by using the 
unknotting assay, and the activity of Topo I was determined by 
using the relaxation assay. Our results indicated that genistein 
inhibited the activity of Topo II in a dose-dependent manner 
(Fig. 5). In the presence of 2 units of Topo II, genistein at 30 
μg/ml inhibited about one-half of Topo II activity (Fig. 5, top, 
Lane 6), while a concentration of 100 μg/ml resulted in a 
complete inhibition (Fig. 5, top, Lane 7). In comparison, com-
plete inhibition of Topo II activity required a concentration of 
200 μg/ml of novobiocin (Fig. 5, top, Lane 2). On a molar basis, 
360 μM genistein or 315 μM novobiocin were required to inhibit 
the Topo II activity. The inhibitory effect of genistein on Topo 
II activity was inversely related to the amount of the purified 
enzyme; in the presence of 20 units of Topo II, complete 
inhibition of enzyme activity required 10 times more genistein 
than in the presence of 2 units of the enzyme (data not shown). 
Genistein was less effective in inhibiting Topo I activity; a 
concentration of 1000 μg/ml inhibited less than one-half the 
activity when 2 units of Topo I were present in the reaction 
mixture (Fig. 5, bottom, Lane 7). TLCK did not affect either 
Topo II (Fig. 5, top, Lanes 8–12) or Topo I activities (data not 
shown). These results confirm the ability of genistein to inhibit 
the activity of purified Topo II.

Topo II interaction with DNA involves transient double-
stranded breakage followed by rejoining of the two strands. The 
Topo II-DNA complex causing the DNA strand breakage can 
be stabilized by drugs such as m-AMS or VM-26, forming 
what are termed "cleavable complexes" (23). These complexes 
can be detected by the conversion of supercoiled plasmid DNA 
its linear form following denaturation and proteolytic deg-
radation of the DNA-bound Topo II. To determine whether 
genistein can stabilize the Topo II-DNA complex, we analyzed 
its ability to produce linear DNA from supercoiled DNA in the 
presence of Topo II (Fig. 6). Genistein at concentrations of 3– 
1000 μg/ml produced double-stranded DNA breaks as shown

Fig. 5. Effect of genistein on the unknotting activity of purified H1-525 cell, 
Topo II (top), or the relaxation activity of purified Topo I (bottom). Top, 2 units 
of purified Topo II were incubated in the absence (Lane 1) or presence of 200 
μg/ml of novobiocin (Lane 2), or 1, 3, 10, 30, or 100 μg/ml of genistein (Lanes 
3–7), or 1, 3, 10, 30, or 100 μg/ml TLCK (Lanes 8–12). Topo II activity was 
determined by converting knotted P4 phage DNA (K) to its unknotted form (U). 
The phage DNA preparation contained also a small amount of linear form (L). 
Bottom, reaction mixtures contained 2 units of purified Topo I (Lane 2), pUC8 
genistein at 10, 30, 100, 300, and 1000 μg/ml (Lanes 3–7). Lane 1, pUC8 plasmid 
DNA control (no enzyme added). Topo I activity was determined by converting 
supercoiled pUC8 plasmid DNA (S) into its relaxed form (R).
by the appearance of linear DNA (Fig. 6, Lanes 2–7). Linear DNA was not detectable in the absence of genistein (Fig. 6, Lane 1) nor in the absence of Topo II (Fig. 6, Lane 9). In our experiment, ATP did not have a major effect on the production of linear plasmid DNA (Fig. 6, compare Lanes 6 and 8). Thus, genistein, like m-AMSA and VM-26 (Fig. 6, Lanes 11–16), induces Topo II-mediated double-stranded breaks. In contrast, novobiocin, which interacts directly with Topo II, does not stabilize the Topo II-DNA complex, as shown by its inability to induce Topo II-mediated double-stranded breaks (Fig. 6, Lanes 17 and 18). These results indicate that genistein can not only inhibit Topo II activity but also stabilize the Topo II-DNA complex.

Enhancement of Protein-associated DNA Strand Breaks in HL-60 and K-562-J Cells Treated with Genistein. In addition to determining the effect of genistein on purified topoisomerases, we measured Topo I and Topo II activities in nuclear extracts obtained from cells treated with this agent using the relaxation and unknotting assays, respectively. Treatment of either K-562-J or HL-205 cells with genistein at a concentration as high as 150 µg/ml for 16 h resulted in no detectable changes in either Topo I or Topo II activities in the nuclear extracts from these cells. Treatment of the cells with 10 µg/ml genistein for as long as 4 days caused less than a 2-fold decrease in the level of extractable Topo II activity (data not shown). These results indicate that Topo I or Topo II activities in nuclear extracts from K-562-J and HL-205 cells are not effectively altered by treating the cells with genistein. We cannot, however, rule out the possibility that the extraction process results in the elimination of genistein from the nuclear extracts and thus restores the Topo II activity to control levels.

Since Topo II-mediated cleavage in intact cells can be markedly decreased without an associated decline in extractable enzyme activity (7), we measured protein-associated DNA strand breaks in control and genistein-treated K-562-J and HL-205 cells. In both cell types, treatment with 30–200 µg/ml of genistein for only 1 h produced protein-associated DNA strand breaks that increased in a dose-dependent manner, with the DNA from the K-562-J cells being somewhat less susceptible to the damaging effects of genistein (Fig. 7). Treatment of HL-205 cells for 1 h with 100 µg/ml of genistein resulted in DNA damage equal to that induced by 105 rads of γ-rays. The same dose of genistein caused DNA damage in the K-562-J cells that was equivalent to that induced by 51 rads of γ-rays (Fig. 8).

Treatment of HL-205 cells for 24 h with genistein doses ranging from 3 to 30 µg/ml also resulted in protein-associated DNA strand breaks (as determined by the alkaline elution assay) that increased in a dose-dependent manner (Fig. 9). Even though these results are expressed as protein-associated single-stranded DNA breaks under alkaline conditions (pH 12.1), a substantial fraction of genistein-induced DNA breaks are double stranded as determined from parallel neutral elution analysis (pH 7.2) of treated HL-205 and K-562-J cells (data not shown). However, DNA strand breaks were not detected in 100 µg/ml of genistein-treated HL-205 cells when proteinase K was omitted from the lysis solution (under alkaline conditions), confirming that the observed DNA strand breaks were protein associated.

Effect of Genistein on Tyrosine Phosphorylation. To study the inhibitory effect of genistein on tyrosine kinase activity, we incubated solubilized membrane fractions of A-431 cells, which are enriched for the EGFTK, with genistein in the presence and absence of its activator EGF. Our results indicated that genistein inhibited both the basal and EGF-activated EGFTK activity by about 90%. No such inhibition was observed with novobiocin, which is not known to be a tyrosine kinase inhibitor (Table I).

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**Fig. 6.** Effect of genistein on DNA cleavage mediated by purified Topo II. Purified Topo II was incubated with supercoiled plasmid pUC8 in the absence (Lane 1) or in the presence of Topo II inhibitors. Lanes 2–7: 5, 10, 30, 100, 300, and 1000 µg/ml of genistein; Lane 8: 300 µg/ml of genistein in the absence of ATP; Lane 9: 300 µg/ml of genistein in the absence of ATP and 10 µg/ml of m-AMSA; Lanes 11–13: 1, 10, or 100 µg/ml of VM-26; Lanes 17 and 18: 100 or 1000 µg/ml novobiocin; Lanes 10 and 20: linear pUC8 DNA (positive control) and Lane 19: supercoiled pUC8 DNA (negative control). Following proteinase K-SDS treatment, samples were loaded on 0.8% agarose and the linear DNA (L) form was separated from the supercoiled (S) and the relaxed (R) DNA by electrophoresis in Tris-borate-EDTA buffer containing 0.5 µg/ml of ethidium bromide. Results are representative of three similar but independent experiments.

**Fig. 7.** Alkaline elution of DNA from HL-205 and K-562-J cells incubated with different concentrations of genistein. Cells containing [3H]DNA were treated with genistein for 1 h and lysed on polycarbonate filters in the presence of proteinase K, and the eluted radioactivity was plotted against time of elution. Treatments were as follows: no treatment (O), 30 µg/ml (●), 100 µg/ml (△), and 200 µg/ml (▲) of genistein. The variation of the data was within 5% among two independent experiments, each performed in duplicate.
HL-205 cells. Moreover, the extent of the reactivity of the anti-phosphotyrosine antibody with two of the proteins changed markedly following genistein treatment.

Discussion

In the present study we report that genistein, an \textit{in vitro} inhibitor of topoisomerases (10) and tyrosine kinases (9), induces a mature phenotype in the human myeloid HL-205 (13) and erythroid K-562-J (14) leukemia cells. Differentiation in the HL-205 cells was characterized by an increase in the percentage of cells reacting with the OKM1 monoclonal antibody directed to a myeloid cell surface antigen (21), positive staining for NSE (22), a characteristic common to monocytes, and expression of NBT dye reduction (16), a marker of granulocytic maturation in these cells. Although the reactivity with the OKM1 antibody was observed in more than 95% of the HL-205 cells, the percentage of cells exhibiting lineage-specific (monocytic or granulocytic) markers did not exceed 50%. Thus, it is not clear whether these markers are expressed by the same or different cell fractions in the HL-205 cells. The expression of the lineage-specific markers may be related to the effect of genistein on a specific biochemical event; for example, inhibition of Topo II activity can result in one phenotype, and inhibition of tyrosine kinase can yield the other phenotype. In this context, the optimal level of genistein (10 \mu g/ml) induced a mature phenotype in only about 50% of the K-562-J cells. Higher doses of the agent caused a suppression of this induction.

The generation of DNA strand breaks is an early event in the genistein-treated HL-205 and K-562-J cells because it is observed as early as 1 h after treatment. Moreover, in spite of this short treatment, these breaks were detected at doses that are either similar or somewhat higher (3-fold) than those required to produce the mature phenotype. These genistein-evoked DNA strand breaks may be linked to the induction of differentiation because they were also observed in other types of cells induced to differentiate by a variety of chemical agents (17, 25–29).

Little or no decrease in Topo II activity was observed in nuclear extracts from cells treated with genistein, which inhibits the catalytic activity of Topo II \textit{in vitro}. This lack of effect may be due to drug removal during the preparation of the nuclear extracts. In common with other Topo II-targeting drugs (e.g., toposide, teniposide, m-AMSA, and 9-hydroxy-ellipticine), genistein may stabilize the complex between Topo II and DNA (the cleavable complex) (23, 30–32), which prevents the rejoining of the two DNA strands following the Topo II-mediated transient double-stranded breakage. Further support for this similarity in mode of action comes from an experiment in which Chinese hamster lung cells selected for resistance to 9-hydroxy-ellipticine also exhibited resistance to genistein (33). However, no definite conclusions can be drawn as to whether the double-stranded breakage in genistein-treated intact cells is indeed mediated through a Topo II mechanism. No matter how the DNA damage is caused, the resulting changes in chromatin structure (34), perhaps combined with inhibition of some tyrosine kinases, may lead to changes in specific cellular programs that cause the acquisition of the mature phenotype in the HL-205 and K-562-J cells. However, additional studies will be required to establish the exact nature of the biological targets of genistein action that results in the acquisition of the mature phenotype in these cells.

In brief, our results indicate that genistein can induce maturation markers in HL-205 and K-562-J cells. This induction is associated with the formation of DNA strand breaks, which may be a factor leading to the acquisition of the mature phenotype.

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References


2623


