# Genistein as an Inducer of Tumor Cell Differentiation: Possible Mechanisms of Action (43841)

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*Abstract.* **Decreased activity of either topoisomerases or tyrosine kinases has been implicated in the differentiation of a number of cell types. It is therefore conceivable that genistein, because of its reported ability to inhibit these activities *in vitro*, may be an inducer of cellular differentiation. We investigated this possibility in human promyelocytic HL-60 and erythroid K-562 leukemia cells and in human SK-MEL-131 melanoma cells. Our results indicated that genistein, in a dose-dependent manner, inhibited cell multiplication and induced cell differentiation. The maturing HL-60 cells acquired granulocytic and monocytic markers. The differentiating K-562 cells stained positively with benzidine, which indicates the production of hemoglobin, an erythroid marker. Following genistein treatment, maturing SK-MEL-131 melanoma cells formed dendrite-like structures and exhibited increased tyrosinase activity and melanin content. Experiments were designed to identify the molecular mechanism of genistein's action. Data from our laboratory suggest that this isoflavone triggers the pathway that leads to cellular differentiation by stabilizing protein-linked DNA strand breakage. Other possible mechanisms reported in the literature are discussed.**

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Two major strategies are currently being used in the fight against cancer. The first relies on destroying tumor cells by taking advantage of some of the intrinsic shortcomings of rapidly proliferating cells. This approach involves the use of drugs, such as adriamycin and etoposide (VP-16), that inhibit certain steps in the replication machinery of cells. The second strategy uses noncytotoxic concentrations of agents that promote terminal differentiation of human tumor cells (1). The therapeutic use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia (2) is an example of this approach, which is gaining momentum not only in chemotherapy but also in cancer prevention with chemical agents (chemoprevention).

Agents that function as inducers of cell differentiation belong to a variety of chemical classes and modulate a diversity of cellular processes. Many such inducers are either ligands that alter signal transduction processes or are inhibitors of enzymes whose action promotes cell proliferation. Protein tyrosine kinases (PTKs), including those coded for by oncogenes such as the *Src* gene family (3) belong to this class of enzymes; increased activity of PTKs are known to give cells a proliferative advantage. Genistein is an inducer of terminal differentiation in human tumor cells (4) that was recognized initially as a phytoestrogen and was later recognized as a PTK inhibitor. Genistein inhibits purified epidermal growth factor (EGF) receptor and $pp60^{v\text{-}src}$ PTK with an $ID_{50}$ of 6–7 μg/ml and inhibits intact human A431 PTK with an $ID_{50}$ of 40 μg/ml (5, 6). Later, it was found that genistein is also an effective inhibitor of eukaryotic DNA topoisomerase (topo) II (4, 7, 8) an enzyme involved in cellular replication.

Evidence is accumulating that populations con-

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suming soybeans have reduced incidence of certain forms of cancer (9). Initial animal studies performed by Barnes *et al.* (10) suggest that two isoflavones, genistein and daidzein, may be the ingredients of soybeans that function as chemopreventive agents. Because of the potential clinical uses of genistein, it is becoming essential to acquire a better understanding of the agent's mode of action at the molecular level.

Following the identification of this flavonoid's effects in cell-free systems, the next logical step is to evaluate its intracellular effects in tumor cell lines. A common response of tumor cells to treatments with subcytotoxic concentrations of genistein is reduced cell proliferation and expression of differentiation markers (4). The sequence of biochemical events underlying this response, however, is poorly understood. In this article, we will describe the differentiation-inducing effects of genistein in human tumor cells and will analyze the available data on its molecular mode of action in relationship to this differentiation.

## Induction of Cell Differentiation by Genistein

We evaluated three human tumor cell lines for their ability to undergo differentiation in response to genistein treatments: the promyelocytic (HL-60) leukemia, myelogenous (K-562) leukemia, and SK-MEL-131 melanoma cell lines. Genistein reduced the proliferation of these cells in a time- and dose-dependent manner (0–7 days, 1–25 μg/ml). Optimal genistein concentrations (which cause a 20%–50% reduction in the number of cells) were chosen for each cell line: 10 μg/ml for HL-60 and K-562 cells and 15 μg/ml for SK-MEL-131 melanoma cells. Higher concentrations or incubations longer than 7 days were cytotoxic. Differentiation in the HL-60 cells was determined by measuring nonspecific esterase activity (NSE), a characteristic marker of the monocytic lineage (11); nitroblue tetrazolium (NBT) dye reduction, characterizing cells maturing towards the granulocytic lineage (12); and reactivity with the OKM1 monoclonal antibody, which detects a cell surface antigen common to both granulocytes and monocytes (13). K-562 terminal cell differentiation was monitored by determining the percentage of hemoglobin-producing cells with the benzidine assay (14). Markers of cell differentiation were measured daily after the second day of treatment. Melanoma cell differentiation was monitored by measuring tyrosinase activity (an early step in melanin biosynthesis), by determining melanin content (15), and by the appearance of a network of dendrite-like structures (Fig. 1). The data summarized in Table I show the expression of the differentiation markers in the HL-60, K-562, and SK-MEL-131 cells after 6 days of treatment with genistein. Clearly, these human tumor cells undergo terminal differentiation in response to the treatment. The HL-60 cells seem to differentiate towards either one of the two possible pathways, but the granulocytic marker NBT was expressed by almost twice as many cells as the monocytic marker NSE. Thus, about 60% of the cells appear to differentiate towards the granulocytic pathway and about 30% towards the monocytic pathway. OKM1-positive cells, representing both lineages, comprise about 90% of the total. In the treated K-562 cell line, about 60% contain hemoglobin, as evidenced by positive staining with benzidine. Differentiation in melanoma cells treated with genistein for 6 days was characterized by an 8-fold increase in tyrosinase activity and a 3-fold increase in melanin content, relative to control cells. The treated cells also exhibit dendrite-like structures typical of mature melanocytes (Fig. 1).

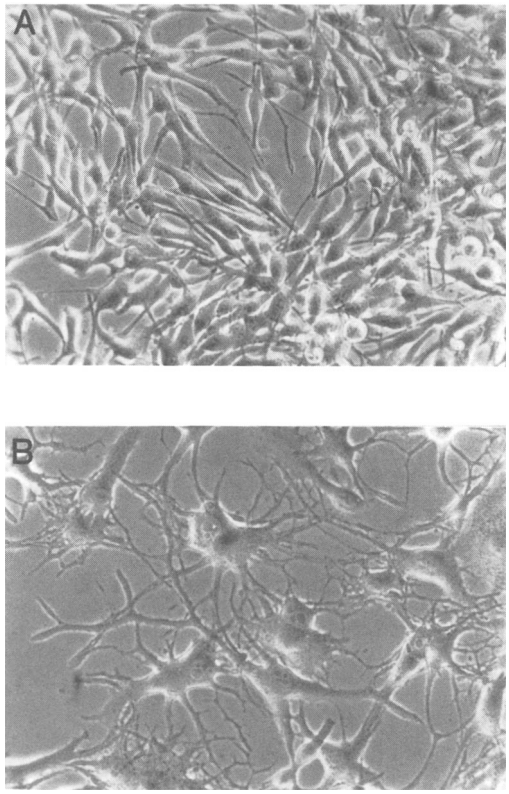


**Figure 1.** SK-MEL-131 cell morphology, visualized by light microscopy, after 6-day culture in the absence (A) or presence (B) of 15 μg/ml genistein.

Following our initial reports (4, 15), several other laboratories also have shown that genistein is an inducer of cell differentiation in a variety of cell types. Table II summarizes, in chronological order, the available studies in which genistein has been reported to induce cell differentiation *in vitro*.

## Possible Mechanisms of Action

The mechanism by which genistein and other flavonoids mediate their cellular effects remains elusive. In 1990, Honma *et al.* (16) compared the effect of eight protein kinase inhibitors in K-562 cells. Only tyrosine

**Table I.** Expression of Maturation Markers in Human Tumor Cells After Treatment with Genistein

| Cell type | Marker[a] | Control[b] | Genistein-treated[c] | Unit |
|---|---|---|---|---|
| HL-60 | NBT | 4 | 58 | Percentage positive |
| | NSE | 4 | 31 | Percentage positive |
| | OKM1 | 7 | 92 | Percentage positive |
| K-562 | Benzidine | 3 | 56 | Percentage positive |
| SK-MEL-131 | Tyrosine | 0.4 | 3.2 | nmol/hr/$10^8$ cells |
| | Melanin | 8.2 | 24.0 | μg/$10^6$ cells |

[a] Expression of differentiation marker was determined 6 days after treatment with genistein.
[b] Control cell cultures were treated with the solvent (DMSO).
[c] The final concentration of genistein was 10 μg/ml in the case of HL-60 and K-562 cells and 15 μg/ml in the case of SK-MEL-131 cells.

**Table II.** Cell Types Reported to Undergo Differentiation After Treatment with Genistein and its Suggested Mode of Action

| Cell type | Lineage (differentiation markers) | Proposed mode of action | Reference |
|---|---|---|---|
| Human myeloid leukemia; HL-60, HL-205, HL-525 | Myelomonocytic (NBT, NSE, OKM-1) | Protein-linked DNA damage/dynamic changes in chromatin structure | 4 |
| Human myeloid leukemia; K-562 | Erythroid (benzidine) | Protein-linked DNA damage/dynamic changes in chromatin structure | 4 |
| Human melanoma; HO, AY-305, C-32, SK-MEL-24, SK-MEL-131 | Melanocytic (melanin, tyrosinase, CF21) | Protein-linked DNA damage/dynamic changes in chromatin structure | 15 |
| Human myeloid leukemia; K-562 | Erythroid (benzidine) | Inhibition of c-*abl* tyrosine kinase activity | 16 |
| Mouse erythroleukemia; MEL | Erythroid | Phosphorylation/dephosphorylation | 18 |
| Mouse megakaryoblastic cell line; C1 | Megakaryocytes (acetylcholinesterase) | Inhibition of v-*abl* tyrosine kinase activity | 17 |
| Human myelomonocytic leukemia ML-1, HL-60, U-937 | Granulocytic (NBT, lysozyme) | Inhibition of phosphatidylinositol turnover | 19 |
| Mouse embryonal carcinoma; F9 | Endoderm (tissue plasmin activator, laminin) | Topo-mediated DNA breaks/alteration in chromatin structure | 20 |
| Rat pheochromocytoma; PC12 | Neuronal (neurite length) | Protein tyrosine kinase inhibition | 21 |

kinase inhibitors (genistein, herbimycin) induced erythroid differentiation as monitored by the benzidine assay. Furthermore, treatment of the cells with c-*abl* antisense RNA also induced benzidine-positive cells. It was concluded that inhibition of a tyrosine kinase is closely related to the induction of erythroid cell differentiation. Similar conclusions were drawn in the case of the mouse C1 megakaryoblastic cells, which express high levels of the v-*abl* gene and of tyrosine kinase activity (17).

Watanabe *et al.* (18) found induction of erythroid differentiation in the mouse erythroleukemia (MEL) cells by genistein. Although no specific mechanism was evident from their data, these investigators seem to favor the possibility that an alteration in protein phosphorylation or dephosphorylation (PKC- or PTK-mediated) may be an early event in the reaction cascade leading to the erythroid differentiation.

Makishima *et al.* (19) reported that genistein induced in the human ML-1, HL-60, and U937 leukemia cells to undergo granulocytic differentiation, but they attributed this to the inhibition of phosphatidylinositol turnover. This conclusion was based mainly on the observation that psi-tectorigenin, which induced cell differentiation, inhibits phosphatidylinositol turnover but not tyrosine kinase activity.

Kondo *et al.* (20) compared the effects of genistein, camptothecin, VM-26, and retinoic acid in mouse embryonal carcinoma (F9) cell differentiation. All of these agents were effective in inducing these

cells to differentiate into parietal endoderm-like cells. Furthermore, genistein and the other two topo inhibitors (camptothecin and VM-26) were able to induce differentiation in a cell line that was resistant to retinoic acid-induced differentiation. These authors suggested that topo inhibitor-induced differentiation involves different steps from those functioning in retinoic acid-induced differentiation. The dynamic changes introduced to chromatin structure due to the stabilization of topo II-DNA complex by genistein (and the other topo II-targeting agents) were considered to be a possible mechanism.

Miller *et al.* (21) found that genistein at a concentration of 370 μ*M* (which is equal to about 120 μg/ml) increased the rate of neurite elongation in PC12 cells that have been previously primed with nerve growth factor (NGF). Genistein was ineffective in cells that were not previously induced to differentiate with NGF. Tyrphostin and herbimycin A, two other tyrosine kinase inhibitors, produced similar effects. Daidzein, which is devoid of tyrosine kinase inhibitory effects, was ineffective in this system. Because a decrease in the steady-state levels of phosphotyrosine-modified proteins was evident in genistein-treated cultures, it was concluded that the inhibition of a tyrosine kinase may be responsible for the effects of genistein. The requirement of such a high dose of genistein in this study may indicate that these cells contain very low levels of topo II activity, which is a reasonable expectation in nondividing cells (22, 23). Cells containing low or nondetectable levels of topo II can tolerate higher levels of topo II-targeting agents because DNA breakage is generally proportional to the cellular levels of the topo II (24). It is not surprising, then, that other enzymes are inhibited at these high genistein concentrations. Monitoring only one or two enzymatic intracellular activities is a major limitation of the above studies, especially, with an agent known to inhibit different enzymes in cell-free systems.

In our effort to identify the mechanism of action of genistein, we examined three variables—nuclear topo II activity, peptide patterns of phosphorylation on tyrosine residues, and DNA breakage (4, 15)—in the HL-60 cell variants K-562 and SK-MEL-131 at different times after treatment with genistein. No changes in nuclear topo II activity (as determined by the P4 unknotting assay) were evident. Changes in the phosphotyrosine peptide patterns, detected in immunoblots with the PY-20 antibody, were evident in the K-562 cells but not in other cell types. There was mainly a reduction in a phosphopeptide of $M_r$ 210,000, believed to be the *bcr/abl* fusion protein generated by the Philadelphia chromosome translocation. This fusion protein is found in cell lines of myelogenous leukemia origin and is known to contain very high levels of PTK activity. The reduction in the phosphotyrosine levels of the $M_r$ 210,000 protein's levels, however, was not evident until 2 days after treatment with 20 μg/ml of genistein.

The earliest and most consistent change, observed in every cell type we examined, was the generation of protein-linked DNA strand breakage. Both single- and double-strand breaks were evident as determined by the alkaline and neutral elution assays respectively (25). Extensive DNA breakage was evident after 24 hr of treatment with the same concentration required to induce differentiation (10–15 μg/ml). DNA breaks were evident even after 1 hr of treatment with higher concentrations. In Figure 2, the effect of genistein in a representative experiment after 1 hr treatment with 30 μg/ml of the agent is shown. When compared with other topo II inhibitors, genistein was less potent than VM-26 and mAMSA in inducing protein-linked DNA strand breaks in HL-60 cells.

The effect of genistein in inducing DNA strand breakage was also examined in a cell-free system using purified topo II and supercoiled plasmid pUC8 DNA as the substrate (Fig. 3). In this assay, the formation of a covalent complex between topo II and DNA is revealed by denaturing the enzyme (with proteinase K-SDS treatment) and analyzing the reaction products with agarose gel electrophoresis. The appearance of linear DNA indicates the ability of the agent to generate double-stranded DNA breakage in the presence of the enzyme. Genistein at the low concentrations of 3 μg/ml produced visible linear DNA (form II). Maximal breakage was evident at 100 μg/ml. The effects of VM-

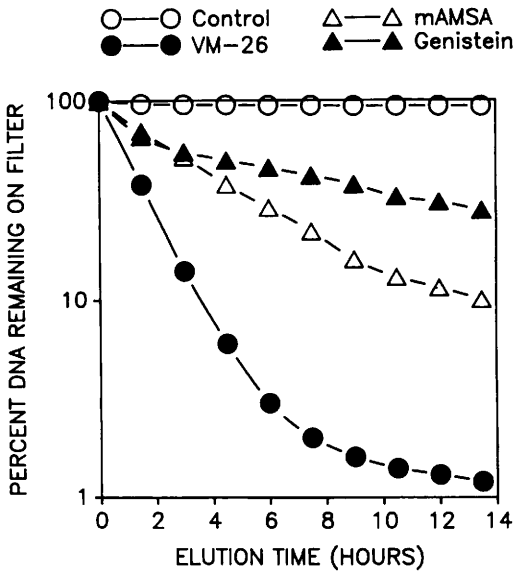


**Figure 2.** Alkaline elution of DNA from HL-60 cells treated with topo II-targeting agents genistein at a final concentration of 30 μg/ml, mAMSA at 1 μg/ml, or VM-26 at 0.1 μg/ml. Cells containing [$^3$H]DNA were treated for 1 hr and lysed on polycarbonate filters in the presence of proteinase K. The eluted radioactivity was determined by scintillation counting and the percentage of DNA remaining on filter was calculated and blotted against time of elution.

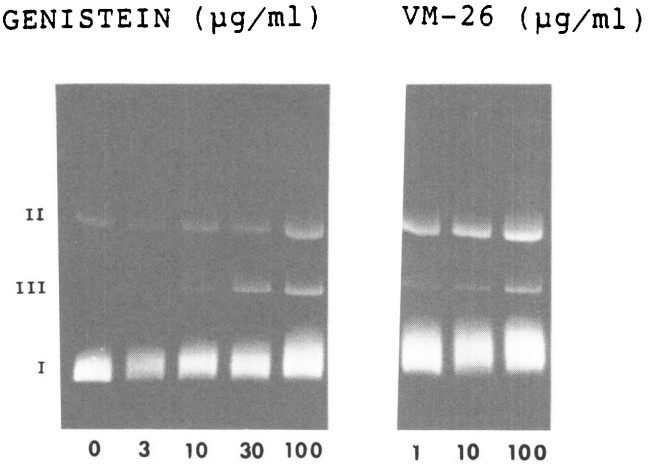


**Figure 3.** Effect of genistein and VM-26 on DNA cleavage mediated by purified topo II. The enzyme was incubated with supercoiled plasmid pUC8 in the presence of increasing concentrations of genistein (left panel) or VM-26 (right panel). Following proteinase K-SDS treatment, samples were loaded on 0.8% agarose gel and the linear DNA (form III) was separated from the supercoiled (form I) and the nicked (form II) DNA by electrophoresis in TBE buffer containing ethidium bromide.

26 are shown for comparison. Neither genistein nor VM-26 produced linear plasmid DNA in the absence of topo II. These results suggest that the DNA breaks observed in the three cell lines following genistein treatment are associated with topo II activity.

Inhibitors of eukaryotic topo II, depending on the stage of the catalytic cycle they inhibit, can be placed into two classes. In the first class belong cytotoxic agents known as topo II-targeting agents or poisons (26), which apparently stabilize the covalent enzyme-DNA complex known as the cleavage complex by preventing the religation step of the reaction (27, 28). Teniposide (VM-26), etoposide (VP-16), ellipticine, doxorubicin, and mAMSA are some representative topo II-targeting agents (16–18). In the second class belong agents which do not stabilize the covalent enzyme-DNA complex but rather prevent formation of this complex and consequently enzymatic turnover. These have been referred as topo II antagonists, because they oppose both the normal topo strand-passing reaction and the action of topo II-targeting agents (29). Novobiocin (30), merbarone (31), aclarubicin (32), fostriecin (33), bis(2,6-dioxopiperazine) derivatives (34), and gossypol (35) are some representative topo II antagonists. Topo II (and topo I) targeting agents are more commonly used as chemotherapeutic agents (36). Based on this information genistein can be classified as a topo II-targeting agent; its mode of action in inhibiting topo II is schematically represented in Figure 4 (A–E).

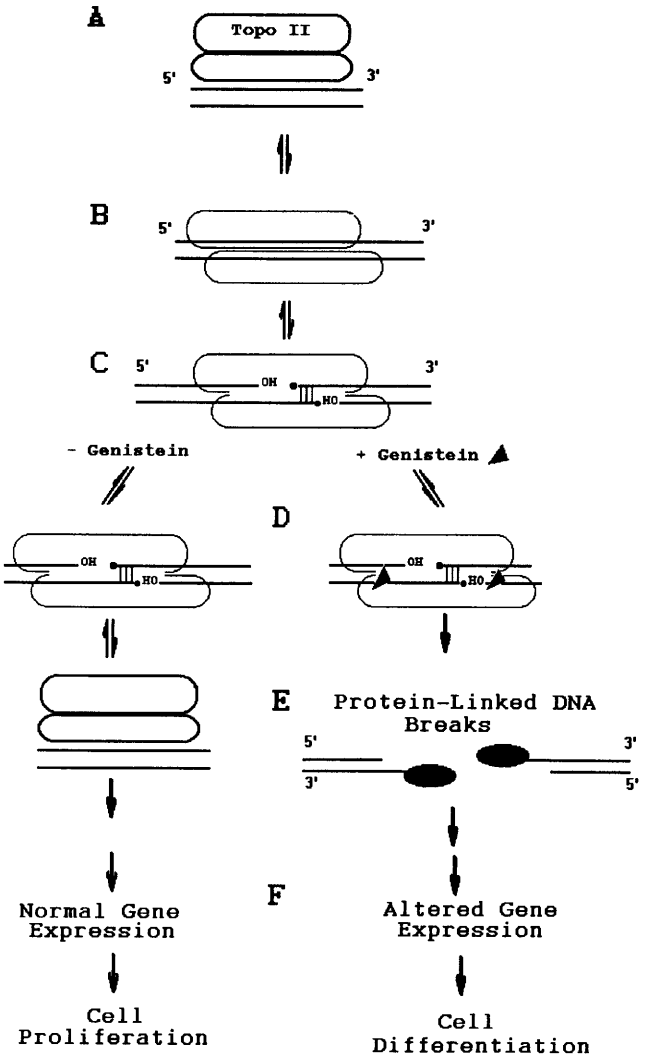


**Figure 4.** Hypothetical mechanism for the stabilization of topo II-mediated DNA breakage by genistein and the induction of tumor cell differentiation. Eukaryotic topo IIα, a homodimer (A) binds to DNA initially noncovalently (B), and later covalently (C). This topo II-DNA covalent complex (normally transient) can be stabilized by topo II-targeting agents (D). Treatment of cells with denaturing agents (proteinase K-SDS) reveals double strand DNA breaks with topo II linked to the 5′ end of each strand (E). These breaks may cause chromatin alterations (F), altered gene expression and cell differentiation (G). In the absence of genistein (left panel D–G), the topoisomerization reaction goes to completion and cell division continues normally allowing the cells to proliferate.

## Conclusions

An altered phosphorylation of regulatory enzymes involved in the signal transduction pathway is expected to have severe biological consequences, including altered cell replication and differentiation. Such regulatory enzymes include the c-*abl*, a proliferation-promoting enzyme with increased PTK activity; the $p34^{cdc2}$ kinase, known to control cell entrance into mitosis; and S6 kinase which modulate protein and RNA synthesis. The reported suppression of HL-60 cell progression through the G2 phase (37) suggests that enzymes such $p34^{cdc2}$ kinase may be affected by genistein directly or indirectly. This effect of genistein on the cell cycle is similar to that of a variety of topo II-targeting agents including VP-16 and mAMSA that

have been shown to stabilize cells into G2 (38, 39). Available evidence suggests that other protein kinases involved in chromatin condensation may be affected by genistein (37). On the other hand, genistein, like other topo II inhibitors, has been shown to stimulate unique DNA strand breaks in the presence of purified DNA substrates such as pBR322 or pUC8 but also with chromosomal DNA *in vivo* (7, 15). Similarly genistein, like other topo II-targeting agents, has been shown to induce apoptosis in HL-60 and human thymocyte cells (38, 40). This observation is not surprising, because topo II-mediated breaks have been proposed to be early events in the pathway leading to apoptosis (41).

Two sets of biochemical events were evident in the above studies. First, genistein interacted with topo II and stabilized the complex between DNA and the enzyme. Second, this flavonoid, directly or indirectly as a PTK inhibitor, affected the phosphorylation state of protein substrates. We previously suggested that changes in chromatin structure brought about by topo II inhibitors may be an early event in the reaction cascade leading to cell differentiation in HL-205 cells (a clonal variant of HL-60 cells), K-562 cells, and several melanoma cell lines. Kondo *et al.* (20) were driven to the same conclusion from their studies in mouse embryonal carcinoma cells (F9). One possible scenario is that protein-linked DNA damage (possibly topo II mediated) results in changes in chromatin configuration which may induce specific changes in gene expression that favor the expression of genes involved in cell differentiation.

Although DNA damage may be necessary for initiating the cascade of events leading to cell differentiation, it may not be sufficient. Different mechanisms may be at work in different cell types. For example, in cell lines of myelogenous leukemia origin such as K-562, which contain activated tyrosine kinase, the inhibition of tyrosine kinase may be a key component and apparently sufficient for differentiation induction, according to the antisense studies of Honma *et al.* (16, 17). In HL-60 and melanoma cells (where low levels of tyrosine kinase activity are detectable), other variables, such as PKC isoforms and enzymes controlling the cell cycle or DNA repair, may be additional components in the cascade of events leading to cell differentiation. These possibilities are shown diagrammatically in Figure 4. Steps A–E are well documented, but Step F is a black box that may contain events such as alterations in chromatin structure, protein phosphorylation, or activity of cell-cycling enzymes.

In the cascade of events leading to cell differentiation, DNA strand breakage may be an early event, and subsequent events may depend on the genetic make-up and the specific enzyme pattern being expressed in the various cell types. The presence or absence of certain DNA repair enzymes, the types and levels of topo II isoforms being expressed, and the types and levels of PTK or other protein kinases are some of the factors that could influence the biological response of a specific cell type.

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