Induction of *in Vitro* Differentiation of Mouse Erythroleukemia Cells by Genistein, an Inhibitor of Tyrosine Protein Kinases

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**ABSTRACT**

We have found that genistein, a specific inhibitor of tyrosine protein kinases, induces *in vitro* erythroid differentiation of mouse erythroleukemia cells. Characterization of the induction process indicated that the genistein-induced differentiation is different from that induced by conventional inducing agents such as dimethyl sulfoxide or hexamethylene-bisacetamide. This conclusion was based upon the earlier appearance of differentiated cells, insensitivity to a specific inhibitor (dexamethasone), and responsiveness of some of the differentiation-resistant cells to genistein in the genistein-induced erythroid differentiation. Possible biological significance of this finding is discussed with respect to the involvement of protein phosphorylation (or dephosphorylation) in mouse erythroleukemia cell differentiation.

**INTRODUCTION**

MEL cells have been known to differentiate into erythroid cells *in vitro* in response to a number of compounds, including DMSO (1), HMBA (2), butyric acid (3, 4), and others (5). In a previous paper, we reported that herbimycin A, a specific inhibitor of mammalian tyrosine protein kinases (6), is an effective inducer of terminal differentiation in MEL cells (7). This was the first demonstration of a compound with known biochemical activity triggering erythroid differentiation. One of the striking features of herbimycin A as an inducer of cell differentiation is that the drug also induced differentiation of mouse embryonal carcinoma (F9) cells and K562 human leukemic cells (7, 8), which are usually induced by retinoic acid and hemin, respectively, but not by DMSO (or HMBA) (9-11). These results suggested the presence of a common step in the differentiation cascades of these cells, which had been considered to be quite different from each other.

In subsequent studies, we examined the effect of other inhibitors of tyrosine protein kinases on erythroid differentiation in MEL cells (12). At relatively low drug concentrations, genistein (13) or ST638 (14) induced differentiation when the drug was added to the culture medium together with agents which block DNA replication, such as mitomycin C, further supporting the notion that inhibition of protein phosphorylation at tyrosine residues triggers, or at least makes the cell susceptible to, differentiation. In this connection, there has been much experimental evidence supporting the notion that PKC is involved in the differentiation of MEL cells. For example, a vincristine-resistant MEL cell line, which exhibits higher levels of PKC-ß activity, was highly sensitive to induction by HMBA (15, 16).

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: MEL, mouse erythroleukemia; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; FCS, fetal calf serum; DMEM, Dulbeco's modified Eagle medium; PMA, phorbol 12-myristate-13-acetate; HMBA, hexamethylenebisacetamide; DMSO, dimethyl sulfoxide.

In a more direct approach, Melloni *et al.* (17) recently reported that introduction of a purified PKC-ß into MEL cells accelerated induced differentiation of MEL cells. Although PKC phosphorylates serine and threonine residues on proteins, these studies and our previous experiments, which suggested the involvement of protein phosphorylation (and dephosphorylation) in cell differentiation, prompted us to further investigate the effect on MEL cell differentiation of inhibition of protein phosphorylation at tyrosine residues by genistein.

In this paper, we report that genistein alone, if added at higher concentrations, induces erythroid differentiation in MEL cells. We have characterized the genistein-induced erythroid differentiation and found that it differs from the differentiation induced by conventional inducing agents, such as DMSO or HMBA, in several aspects, including kinetics of the appearance of erythroid cells, insensitivity to a specific inhibitor (dexamethasone), and others. The significance of the findings is discussed with respect to the involvement of protein phosphorylation (or dephosphorylation) in MEL cell differentiation.

**MATERIALS AND METHODS**

Materials. Genistein was obtained from Funakoshi Co. (Tokyo, Japan). HMBA and PMA were gifts from T. Yamane (Bell Laboratories, Murray Hill, NJ) and M. Terada (National Cancer Institute, Tokyo, Japan), respectively. Dexamethasone was obtained from Sigma (St. Louis, MO). These reagents were first dissolved and stocked in the following solvents and concentrations and then appropriately diluted with H₂O when used: genistein (in DMSO), 100 mg/ml; HMBA (in H₂O), 1 mM; PMA (in acetone), 1 mg/ml; and dexamethasone (in ethanol), 10 mg/ml.

Eagle's minimal essential medium and DMEM were purchased from Nissui Seiyaku (Tokyo). FCS was obtained from Sigma. Rabbit anti-mouse hemoglobin antibody was purchased from Cappel Laboratories (West Chester, PA). Mouse monoclonal anti-phosphotyrosine antibody and sheep anti-mouse IgG horseradish peroxidase-conjugated antibody were purchased from ICN (Mesa, CA) and Amersham, respectively.

Cells and Cell Culture. MEL (Friend) cells (745A.DS19) were supplied by M. Terada (National Cancer Institute, Tokyo). Differentiation-resistant MEL cells were isolated in this laboratory after treatment of the cells with N-nitro-1,N'-nitrosoquainidine (18). The cells were cultured at 37°C in a CO₂ (5%) incubator in MEM supplemented with FCS (12%, v/v). For the assay of colony-forming ability, DMEM was supplemented with FCS (15%, v/v) and methylcellulose (1.5%, w/v).

Assay of Hemoglobin Accumulation. Hemoglobin accumulation in MEL cells was assayed in several ways. For staining of cells which accumulate hemoglobin, we employed the benzidine staining method originally described by Orkin *et al.* (19). For direct staining of protein, the cells were lysed by incubation in Nonidet P-40 (0.5% v/v) at 37°C for 20 min. The supernatant, after centrifugation, was diluted with an equal volume of 2x SDS-PAGE sample buffer containing 125 mM Tris-HCl, pH 6.8, 4.6%, w/v, SDS, 20%, v/v, glycerol, 0.02%, w/v, bromophenol blue, and 2%, v/v, 2-mercaptoethanol (20) and boiled at 100°C for 5 min. Samples (5 μg protein/lane) were subjected to SDS-PAGE (20%, w/v), and proteins were silver stained using a staining kit (Daiichi Chemical, Tokyo). Immunoblot using a staining kit (Daiichi Chemical, Tokyo).
Chemical, Tokyo). Immunoblot analysis of hemoglobin was performed as follows. The boiled supernatant (see above) (10 μg protein/lane) was electrophoresed through a polyacrylamide gel (15%, w/v) and transferred (electroblotted) to a nitrocellulose filter (0.45 μm; Promega, Madison, WI), using a buffer containing 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% (v/v) methanol, at 4°C for 2 h. Hemoglobin was detected with rabbit anti-mouse hemoglobin antibody (20 μg/ml; Cappel), using a Proto Blot kit (Promega).

Assay of Colony-forming Ability of MEL Cells. MEL cells cultured in minimum essential medium with FCS were diluted with fresh medium to 2 x 10^5 cells/ml and incubated in plastic dishes (24 wells, each 1.0 ml; Falcon), in the presence or absence of genistein, in a CO2 incubator at 37°C. After 5 days, the cells were washed twice with phosphate-buffered saline to remove the drug and then plated on a semisolid medium (DMEM with 15%, v/v FCS and 1.5%, w/v, methylcellulose) in plastic Petri dishes (60-mm diameter) after appropriate dilution, which gave approximately 1000 cells/dish. After incubation for 2 weeks at 37°C in a CO2 incubator, the number of colonies was counted.

Western Blotting. The cells (~2 x 10^6) were washed with phosphate-buffered saline containing 1 mM Na2VO4 and incubated with 100 μl of RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na2VO4, 1%, v/v, Triton X-100, 1%, w/v, sodium deoxycholate, 0.1%, w/v, SDS, 1 trypsin inhibitor unit/ml aprotinin) for 20 min at 0°C. The lysate was centrifuged at 15,000 rpm for 20 min at 4°C, the supernatant was mixed with 5× SDS-PAGE buffer (20) and boiled for 5 min, and the samples (100 μg protein) each were subjected to SDS-PAGE (10%, w/v, polyacrylamide). After proteins were transferred to Hybond-C (Amersham) with buffer (48 mM Tris base, 39 mM glycine, and 20%, v/v, methanol), phosphotyrosine-containing proteins were detected by using mouse monoclonal anti-phosphotyrosine antibody (0.7 μg/ml; ICN), sheep anti-mouse IgG horseradish peroxidase-conjugated antibody (Amersham), and enhanced chemiluminescence (ECL) detection solution (Amersham).

RESULTS

In order to further investigate the mechanism of differentiation induced by inhibitors of tyrosine protein kinases, we examined the effect of genistein on the induction of erythroid differentiation in MEL cells. Genistein is an isoflavone compound that is isolated from fermentation broth of Pseudomonas sp. and was recently reported to be a specific inhibitor of tyrosine specific kinases (13). We reported previously that, at lower concentrations (~1 μg/ml), genistein induced erythroid differentiation synergistically when the drug was added to culture medium together with mitomycin C or other DNA-damaging agents (12). When relatively high concentrations (5–20 μg/ml) of genistein were used, however, we found that the drug by itself induced erythroid differentiation, which is consistent with a report on Differenol A (later identified to be genistein)-induced differentiation (21), allowing us to compare the differentiation process with that induced by DMSO or HMBA.

As seen in Fig. 1A, at 10 μg/ml or higher concentrations of the drug, approximately 90% of the cells became benzidine positive. Under the same experimental conditions, DMSO (or HMBA) induced 80 to 90% benzidine-positive cells (data not shown). The induction of benzidine-positive cells by genistein was irreversible. When the cells were exposed to genistein for 2 days and incubated in the absence of the drug thereafter, benzidine-positive cells still appeared at high frequency (80%) (Fig. 1B). This suggests that genistein treatment not only triggers hemoglobin accumulation but also causes the cells to commit to differentiation, as generally observed with DMSO- or HMBA-induced MEL cell differentiation (1, 22, 23).

In order to quantitate the level of hemoglobin in the cells induced by genistein, cell-free extracts from cells incubated with the drug were subjected to SDS-PAGE and also to immunoblotting. As seen in Fig. 2, A (SDS-PAGE) and B (immunoblotting), the level of hemoglobin in cells induced by genistein was the same as that in cells treated with DMSO or HMBA.

We also examined whether the appearance of benzidine-positive cells or accumulation of hemoglobin accompanied loss of colony-forming ability, another characteristic of MEL cell differentiation (22, 23). As shown in Table 1, the appearance of benzidine-positive cells induced by genistein (15 μg/ml) accompanied almost complete loss of colony-forming ability, which seemed even more pronounced than that observed in the differentiation induced by DMSO. These results (hemoglobin accumulation, the apparent commitment to differentiation, and the loss of colony-forming ability) observed following genistein treatment were, at least ostensibly, very similar to those observed for erythroid differentiation induced by conventional inducing agents such as DMSO or HMBA.

In contrast to genistein, staurosporine, a specific inhibitor of PKC, exhibited no erythroid-inducing activity. In a wide range of drug concentrations (3 to 100 nm), incubation of MEL cells with the drug did not cause any increase in the number of benzidine-positive cells over the background values (0.1–0.5%) during a 5-day incubation period (data not shown), suggesting that inhibition of protein phosphorylation at tyrosine residues, but not at serine or threonine residues, triggers erythroid differentiation of MEL cells.

In order to obtain a clue to the mechanism of differentiation induced by genistein and other inhibitors of tyrosine protein kinases, we attempted to distinguish the erythroid differentiation induced by genistein from that induced by DMSO (or HMBA). First, we observed that, following exposure to genistein, benzidine-positive cells appeared at least 1 day earlier than after exposure to DMSO, as if the genistein-induced intracellular cascade leading to differentiation consisted of fewer steps than those involved in DMSO (or HMBA)-induced differentiation. Fig. 3 compares the kinetics of the appearance of benzidine-positive cells induced by genistein with that induced by DMSO. As shown in the figure, benzidine-positive cells started to appear after 24 h of exposure of the cells to genistein and reached a maximum after 4 days of incubation. Under the same conditions, DMSO induced benzidine-positive cells after 2 days of incubation and they reached a maximum after 5 days of incubation (Fig. 3).

Second, we found that the genistein-induced differentiation
was resistant to dexamethasone, a known inhibitor of differentiation of MEL cells to erythroid cells (24, 25). As seen in Table 2, while dexamethasone almost completely inhibited DMSO- or HMBA-induced differentiation, as reported before, it had almost no effect on the erythroid differentiation induced by genistein (less than 10% inhibition). On the other hand, PMA, a biologically active phorbol ester and another typical inhibitor of MEL cell differentiation (26, 27), inhibited both DMSO (or HMBA)-induced and genistein-induced differentiation almost equally. This suggests that, whereas the molecular cascade leading to erythroid differentiation by genistein shares a PMA-sensitive step with that triggered by DMSO (or HMBA), genistein apparently bypasses the dexamethasone-sensitive step which is present in DMSO (or HMBA)-induced differentiation. This also suggests that the mechanism of inhibition of MEL cell differentiation by PMA is different from that of inhibition by dexamethasone.

Furthermore, the genistein-treated cells seemed to be morphologically different from those treated with DMSO or HMBA (data not shown). After 2 days of incubation, we observed that the majority (~80%) of the cells became enlarged, even at lower (~5 μg/ml) genistein concentrations.

Previously, we isolated several MEL cell mutants that are resistant to DMSO-induced differentiation (18). The erythroid differentiation in these resistant cells (DMSO-1, DMSO-2, and DMSO-3) was less than 10%, even after prolonged incubation with DMSO (280 mM), as opposed to over 80% differentiation in the control (parental) cells. All of these cell lines were also refractory not only to erythroid differentiation by HMBA but also to the differentiation induced by butyric acid.

### Table 1 Effect of genistein on colony-forming ability

<table>
<thead>
<tr>
<th>Addition</th>
<th>Colony formation (%)</th>
<th>Benzidine-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>92.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Genistein</td>
<td>0.1</td>
<td>92.9</td>
</tr>
<tr>
<td>DMSO</td>
<td>7.2</td>
<td>88.3</td>
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</table>

### Table 2 Effect of dexamethasone and PMA on erythroid differentiation induced by genistein

<table>
<thead>
<tr>
<th>Addition</th>
<th>None</th>
<th>Dexamethasone</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Genistein</td>
<td>90.0</td>
<td>81.9</td>
<td>4.7</td>
</tr>
<tr>
<td>DMSO</td>
<td>95.0</td>
<td>11.1</td>
<td>12.3</td>
</tr>
<tr>
<td>HMBA</td>
<td>92.1</td>
<td>4.7</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Fig. 3. Kinetics of the appearance of benzidine-positive cells. MEL cells were incubated in the presence of genistein (15 μg/ml) or DMSO (280 mM). At the times indicated in the figure, samples were withdrawn and benzidine-positive (B⁺) cells were counted; they are expressed as percentages of the treated cells. In benzidine-positive cells in the presence of genistein. A, benzidine-positive cells in the presence of DMSO. For details, see “Materials and Methods.”

Fig. 2. Accumulation of hemoglobin by genistein. A, accumulation of hemoglobin assayed by SDS-PAGE. MEL cells (2 × 10⁶ cells/ml) were incubated with either HMBA (5 mM), DMSO (280 mM), or genistein (15 or 20 μg/ml) for 5 days, at 37°C in a CO₂ incubator, and were subjected to SDS-PAGE as described in “Materials and Methods.” Lane 1, hemoglobin (standard); lane 2, HMBA-treated cells; lane 3, DMSO-treated cells; lane 4, genistein (20 μg/ml)-treated cells; lane 5, genistein (15 μg/ml)-treated cells; lane 6, untreated (control) cells. Percentages of benzidine-positive (B⁺) cells induced for each sample are also shown at the bottom of each lane. Arrowhead, position of hemoglobin. B, accumulation of hemoglobin assayed by immunoblotting. MEL cells (2 × 10⁶ cells/ml) were incubated in the presence of HMBA (5 mM), DMSO (280 mM), or genistein (20 ng/ml) for 5 days, at 37°C in a CO₂ incubator, and were subjected to immunoblotting, as described in “Materials and Methods.” Lane 1, HMBA-treated cells; lane 2, DMSO-treated cells; lane 3, genistein-treated cells; lane 4, untreated (control) cells; lane 5, hemoglobin (standard). Arrowhead, position of hemoglobin. Percentages of benzidine-positive cells induced for each sample are shown at the bottom of each lane.
or several other known erythroid-inducing agents. When these MEL cell lines were incubated with genistein, however, two of the three mutant cell lines (DMSO'-2 and DMSO'-3) underwent almost normal erythroid differentiation, although one cell line (DMSO'-1) was still resistant to differentiation (Table 3).

We also investigated whether the intracellular levels of particular phosphotyrosine-containing proteins are affected by genistein in these mutant cell lines. The cells (DMSO'-1, DMSO'-2, and 745A) were incubated in the presence of genistein (20 μg/ml) and harvested 24 h later and, after gel electrophoresis, cell-free extracts were subjected to Western blot analysis using antiphosphotyrosine antibody. As seen in Fig. 4, although more or less uniform reduction of most of the phosphotyrosine-containing proteins was observed, the levels of some phosphotyrosine-containing proteins were reduced more drastically than those of others in some of the cell lines. For example, the intensities of two proteins (Fig. 4, arrows A and B) were reduced in DMSO'-2 (Fig. 4, lanes 2 and 3) and wild-type (745A) (Fig. 4, lanes 3 and 6) cells, both of which were responsive to genistein in erythroid differentiation (Table 3). In DMSO'-1 cells, which were resistant to erythroid differentiation by genistein (Table 3), however, the intensities of these bands were apparently not affected by the drug. Besides these, the intensity of another band (Fig. 4, arrow C) was reduced by genistein treatment in DMSO'-1 and DMSO'-2 cells, but not in 745A cells. Although these results must be carefully interpreted with respect to their significance in erythroid differentiation, it is quite clear that genistein treatment affected the intracellular level of specific phosphotyrosine-containing proteins, with the effect being different among differentiation-resistant mutant cell lines.

In summary, with respect to at least four different characteristics (kinetics of the appearance of benzidine-positive cells, insensitivity to dexamethasone, morphology of the cells, and differentiation of some of the differentiation-resistant MEL cells lines), we found that genistein-induced erythroid differentiation can be distinguished from that triggered by DMSO or HMBA.

**DISCUSSION**

In this paper, we have reported that genistein, one of the inhibitors of tyrosine protein kinases, induces erythroid differentiation in MEL cells in vitro. Probably the most interesting conclusion from the results presented in this paper would be that genistein-induced differentiation is apparently different from that induced by DMSO or HMBA. Although erythroid differentiation in MEL cells is induced by a number of inducing agents, the intracellular cascade leading to differentiation mediated by most of the inducing agents has been considered to be essentially the same, with the possible exception of hemin-induced hemoglobin accumulation (28). However, hemin-induced hemoglobin accumulation, which is reversible, is now considered not to represent in vitro erythroid differentiation but to be derived from hemin-induced specific expression of a globin gene (28). The earlier appearance of benzidine-positive cells, the insensitivity to dexamethasone, and the differentiation of some of the differentiation-resistant MEL cells that are reported here and are unique in the genistein-induced differentiation strongly suggest that the genistein-induced intracellular cascade is different, at least partially, from that induced by DMSO (or HMBA). Based upon these results, one could even speculate that the genistein-induced differentiation cascade bypasses the earlier steps present in DMSO (or HMBA)-induced differentiation, thus causing these differences.

How are the results presented here related to the role played by PKC in MEL cell differentiation suggested by the recent experimental results of Mellon et al. (17), most notably the accelerated induction of erythroid differentiation in MEL cells into which PKC-β had been introduced? It is quite obvious that their results do not necessarily contradict our findings presented here and reported previously. As described above, staurosporine, a specific inhibitor of PKC, had no erythroid-inducing activity. It could, therefore, be speculated that PKC, as suggested from its role in signal transduction, is involved in an early stage of the erythroid differentiation cascade and that proteins that are modified (phosphorylated) by the enzyme are, directly or indirectly, involved in the subsequent step involving dephosphorylation of specific phosphorylated proteins, a reaction probably required for the commitment of MEL cells.

So far, all of the known inhibitors of tyrosine protein kinases that have been examined triggered in vitro differentiation not only in MEL cells but also in F9 cells and K562 cells (Refs. 7

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**Table 3** Erythroid differentiation in differentiation-resistant MEL cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Benzidine-positive cells (%)</th>
<th>Genistein</th>
<th>DMSO</th>
<th>HMBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>745A (DS19)</td>
<td>92.7</td>
<td>77.6</td>
<td>80.0</td>
<td></td>
</tr>
<tr>
<td>DMSO'-1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>DMSO'-2</td>
<td>75.9</td>
<td>2.0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>DMSO'-3</td>
<td>85.1</td>
<td>0.0</td>
<td>18.9</td>
<td></td>
</tr>
</tbody>
</table>

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* T. Watanabe, S. Nomura, and M. Oishi, unpublished results.
and 8 and this study). It seems, therefore, that there is a common step in differentiation cascades, at least in these cells, and the step is somehow associated with protein phosphorylation (or dephosphorylation) at tyrosine residues. Our results, of course, are still too premature to provide insight into the molecular mechanism of how the inhibition of protein phosphorylation at tyrosine residues triggers differentiation similarly in these cell lines. We also do not know whether the induction by genistein is caused by a direct effect of inhibition of tyrosine phosphorylation of a specific protein(s) or by a more indirect adverse effect of the drug on cell physiology, stemming from inhibition of tyrosine phosphorylation in proteins. Identification of a cellular protein(s) whose phosphorylation at tyrosine residues is inhibited by both genistein and DMSO (or HMBA) should give some clues to our understanding of this complex biological processes.

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