Induction of Erythroid Differentiation of K562 Human Leukemic Cells by Herbimycin A, an Inhibitor of Tyrosine Kinase Activity

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ABSTRACT

Herbimycin A, a benzoquinonoid ansamycin antibiotic, is found to reduce intracellular phosphorylation by tyrosine protein kinase. The human chronic myelogenous leukemia cell line K562 expresses a structurally altered c-abl protein with tyrosine kinase activity. When K562 cells were induced to undergo erythroid differentiation by hemin, reduction in the intracellular level of tyrosine phosphorylation occurred. In order to understand the relationship between induction of differentiation and reduction of tyrosine phosphorylation by the c-abl gene product, the effect that herbimycin A, a selective inhibitor of intracellular tyrosine kinase activity, exerts on the differentiation of K562 cells was examined. Reduction of tyrosine phosphorylation in K562 cells by herbimycin A was observed within 1 h. Nonsyntoxic concentrations of herbimycin A induced erythroid differentiation of K562 cells but not of murine erythroleukemia 745A cells. The other human myeloid leukemia cell lines (HL-60, THP-1, and U937) tested were not induced to undergo cell differentiation by this antibiotic. Herbimycin A and the other well-known inducers such as hemin, butyric acid, Adriamycin, and 1,3-d-arabinofuranosylcytosine had additive or more than additive effects on induction of erythroid differentiation of K562 cells. With respect to inhibition of cell growth, the sensitivity of K562 cells to herbimycin A was highest in the human leukemia cell lines we tested. Nonsyntoxic concentrations of herbimycin enhanced the antiproliferative effect of Adriamycin or 1,3-d-arabinofuranosylcytosine on K562 cells. Combination therapy with herbimycin A and its derivatives may be considered for use in the treatment of some types of leukemia where tyrosine kinase activities are implicated as determinants of the oncogenic state.

INTRODUCTION

Translocation of the c-abl gene from chromosome 9 to 22, resulting in the Philadelphia chromosome (Ph1), occurs in >90% of CML patients (1-4). A structurally altered c-abl protein (p210c-"**') has been detected in the CML cell line K562 and in leukemic cells of patients with CML accompanying translocation (9;22) (5, 6). The amino-terminal alteration of p210c-"**' in K562 cells activates its tyrosine-specific protein kinase activity, similar to what occurs upon viral transduction of c-abl (5). The transforming activity of the p210c-"**' protein is known to be mediated by its tyrosine kinase activity. The tyrosine kinase activity of p210c-"**' may also promote proliferation and might be responsible for maintaining the CML cells at the blast stage.

Erythroid differentiation of the K562 cell line is induced by various compounds (7-10). When K562 cells were induced with hemin to undergo erythroid differentiation, reduction in p210c-"**' protein synthesis was observed (11). This reduction appears to be specifically related to differentiation, because there is no general reduction in protein synthesis and the synthesis of the normal c-abl protein with a molecular weight of 137,000 is not affected by hemin.

Herbimycin A, isolated from the cultured filtrate of Streptomyces sp. MH237-CF-8, causes reversion of the transformed Rous sarcoma virus-infected rat kidney cell (12) to normal morphology. The antibiotic reduced p60src-associated protein kinase activity in these cells and was also effective against various cells transformed by the other tyrosine kinase oncogenes including v-abl, but it did not reverse the morphology transformed by oncogenes raf, ras, and myc (13). In the present investigation, we examined the effect of herbimycin A on the growth and differentiation of K562 cells.

MATERIALS AND METHODS

Cells and Culture Conditions. The K562 cell line was originally established from a pleural effusion of a patient with chronic myelogenous leukemia in terminal blast crisis (14). Erythroid differentiation of this cell line has been shown with various inducers (8, 10). K562, HL-60 (15), THP-I (16), and U937 (17) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 in air. Friend erythroleukemia (clone 745A) cells (18) were cultured in Eagle's minimum essential medium with 10% fetal bovine serum. Mouse myeloid leukemia M1 cells were cultured as reported previously (19).

Assay of Cell Growth and Erythroid Differentiation. Cells (5 × 10^6/ml) were suspended in 1 ml of culture medium and were cultured with or without herbimycin A at 37°C in a multidiish (Nunc, Roskilde, Denmark). The cell number was counted with a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL) 4 days after treatment. The erythroid differentiation of the cells was scored by benzidine staining by the procedure reported previously (8). Hemoglobin was assayed by a photometric method using benzidine, previously reported by Testa et al. (20). This method allows detection of hemoglobin diluted to less than 5 µg/ml.

Labeling with 32P and Immunoprecipitation. Exponentially growing cells (2 × 10^6/ml) were suspended in RPMI 1640 lacking phosphate and containing 5% fetal bovine serum which had been dialyzed against 0.14 M NaCl and were incubated in the presence or absence of 5 × 10^-8 M herbimycin A. Labeling was started by addition of 32P, (Amersham; 1 mCi/ml) and incubation proceeded for 1–6 h at 37°C. The labeled cells were collected and washed 3 times with cold PBS and then lysed at 4°C by sonication for 1 h. The supernatant was diluted 10-fold with PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonfluoride. The cell extract was clarified by centrifugation at 15,000 × g for 10 min and the supernatant was diluted 10-fold with PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonfluoride. The diluted sample was treated with agarose-linked monoclonal antibody against phosphotyrosine (Oncogene Science, Manhasset, NY) or polyclonal antibody against c-abl (Oncogene Science) for 1 h at room temperature and was incubated overnight at 4°C. Immune complex with anti-c-abl antibody was precipitated with agarose-linked protein A (Zymed Labo, San Francisco, CA). Immunoprecipitates were washed five times in 50 mM Tris-HCl (pH 7.5)-0.5 M NaCl-0.1% SDS-1% Triton X-100-0.5% sodium deoxycholate-1 mM phenylmethylsulfonfluoride buffer and were then analyzed on 4-20% SDS-polyacrylamide gradient gels as described by Laemmli (21) followed by autoradiography with an intensifying screen. The phosphorylation levels were quantitated by densitometric scanning for the autoradiographs.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CML, chronic myelogenous leukemia; p210c-"**', structurally altered c-abl protein with a molecular weight of 210,000; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.
DIFFERENTIATION OF K562 BY HERBIMYCIN

The cells (5 x 10^4 cells) were inoculated in 1 ml of the culture medium in a multidish (24 wells; Nunc, Denmark) and treated with herbimycin A for 4 days.

Fig. 1. Effect of herbimycin on growth and erythroid differentiation of K562 cells. The cells (5 x 10^4 cells) were inoculated in 1 ml of the culture medium in a multidish (24 wells; Nunc, Denmark) and treated with herbimycin A for 4 days. Percentage of benzidine-positive (B*) cells; --- --- ---, cell number.

Table 1 Induction of erythroid differentiation of K562 cells by herbimycin A

<table>
<thead>
<tr>
<th>Herbimycin concentration (x 10^-8 M)</th>
<th>Cell no. benzidine-positive cells</th>
<th>Percentage of benzidine-positive cells</th>
<th>Hemoglobin (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.8</td>
<td>3.0</td>
<td>0.47</td>
</tr>
<tr>
<td>2.5</td>
<td>14.7</td>
<td>11.3</td>
<td>1.66</td>
</tr>
<tr>
<td>5.0</td>
<td>13.4</td>
<td>15.8</td>
<td>2.12</td>
</tr>
<tr>
<td>7.5</td>
<td>9.3</td>
<td>30.3</td>
<td>2.82</td>
</tr>
<tr>
<td>10.0</td>
<td>4.8</td>
<td>51.5</td>
<td>2.47</td>
</tr>
</tbody>
</table>

RESULTS

After incubation for 4 days herbimycin A was found to inhibit the growth of K562 cells at concentrations higher than 6 x 10^-8 M, 50% inhibition of growth occurring at 9.5 x 10^-8 M (Fig. 1). Benzidine positivity is a typical marker of erythroid differentiation. The increase in hemoglobin content roughly paralleled the increase in percentage of benzidine-positive cells (Table 1). Induction of the benzidine-positive cells was dose dependent and could be observed at concentrations of herbimycin A that did not inhibit growth. The maximal differentiation-inducing effect was obtained at 1 x 10^-7 M herbimycin A, which caused about 65% inhibition of cell growth.

Fig. 2. Herbimycin-induced erythroid differentiation and its relationship to commitment. K562 cells were inoculated into culture medium at 5 x 10^4 cells/ml, and 7.5 x 10^-8 M herbimycin A was added on Day 0. In one set of cultures, herbimycin A was washed out on Day 1, and then the cells were cultured without herbimycin A (C). In the other set, herbimycin A was washed out on Day 2, and then the cells were cultured without herbimycin (W). Benzidine-positive (B*) cells were assayed in aliquots of these cells. C, cells in culture with herbimycin; W, cells in culture without herbimycin.

Table 2 Effect of herbimycin A on growth of some leukemia cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inhibition of cell growth (IC50 x 10^-8 M)</th>
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<tbody>
<tr>
<td>Human</td>
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</tr>
<tr>
<td>K562 (erythroleukemia cells)</td>
<td>9.5 ± 0.3 e</td>
</tr>
<tr>
<td>HL-60 (promyelocytic cells)</td>
<td>103 ± 4</td>
</tr>
<tr>
<td>THP-1 (monocytic cells)</td>
<td>113 ± 1</td>
</tr>
<tr>
<td>U937 (monocytic cells)</td>
<td>175 ± 1</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>745A (Friend erythroleukemia cells)</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>M1 (myeloblastic leukemia cells)</td>
<td>14.4 ± 1.3</td>
</tr>
</tbody>
</table>

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Fig. 3. Tyrosine-phosphorylated proteins in K562 cells treated with or without herbimycin. Exponentially growing cells were collected and labeled with 32P, for 3 h in the absence (A) or presence (B) of 5 x 10^-8 M herbimycin A. The cell extracts (3 x 10^7 cpm) were immunoprecipitated with phosphotyrosine monoclonal antibody (I) or anti-c-erb antibody (2), and the resulting immune complexes were analyzed by SDS-polyacrylamide gel electrophoresis. Molecular standards (in thousands): 200K, myosin H-chain; 97.4K, phosphorylase b; 68K, bovine serum albumin.

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Fig. 4. Effect of simultaneous treatment with herbimycin and the other differentiation inducers on the differentiation of K562 cells. The cells were treated for 3 days with (O) or without (•) the other inducers in the presence of herbimycin A. A, 3 x 10^-7 M Adriamycin; B, 3.5 x 10^-8 M 1-β-D-arabinofuranosylcytosine; C, 1 x 10^-6 M hemin; D, 1 x 10^-7 M butyric acid.

When the cells (5 x 10^4/ml) treated with 7.5 x 10^-8 M herbimycin A for 1 day were washed twice with PBS and then resuspended in fresh culture medium without the antibiotic and incubated for 3 days, about 80% of the cells were benzidine positive at Day 4 (Fig. 2). Thereafter, however, they gradually reverted back to the benzidine-negative phenotype (data not shown). The same result was obtained when the cells were...
with 4 x 10^{-8} M herbimycin and 1.1 x 10^{-8} M Adriamycin, the concentration of Adriamycin required for 50% inhibition of cell growth was 1.1 x 10^{-8} M. When K562 cells were treated simultaneously with herbimycin A and the other well-known inducers, cell growth was inhibited by 10-15%. The results show that herbimycin A and Adriamycin or 1-β-D-arabinofuranosylcytosine have an additive or more than additive effect with some well-known inducers.

K562 cells were not induced to differentiate by herbimycin A, although the concentration used in this experiment did not affect the incorporation of [3H]thymidine and [14C]leucine into bulk DNA and protein, respectively (data not shown).

The steady-state levels of tyrosine-phosphorylated proteins in K562 cells were determined by immunoprecipitation with an antibody specific for phosphotyrosine. Immunoprecipitation detected one major tyrosine-phosphorylated protein and several faint bands of tyrosine-phosphorylated proteins (Fig. 3). The molecular weight of the major tyrosine-phosphorylated protein was 210,000. This protein was p210_{c-abl} tyrosine kinase itself (5, 6, 11). This was demonstrated by immunoprecipitation with anti-α-abl antibody (Fig. 3). When K562 cells were labeled with 32P, in the presence of 5 x 10^{-8} M herbimycin A, the level of all tyrosine-phosphorylated proteins was greatly reduced. Early reduction of approximately 55% of p210_{c-abl} could be demonstrated after 1 h incubation with 5 x 10^{-8} M herbimycin A. Tyrosine phosphorylation was barely detected in the cells that had been treated with herbimycin A for 3 h. The herbimycin concentration used in this experiment did not affect the incorporation of [3H]thymidine and [14C]leucine into bulk DNA and protein, respectively (data not shown).

The sensitivity of K562 cells to inhibition of their growth by herbimycin A was greater than that of the other human leukemia cell lines we tested (Table 2). HL-60, THP-1, and U937 cells were not induced to differentiate by herbimycin A, although these cells can be induced to differentiate (10). The Friend erythroleukemia cell line 745A was more sensitive to herbimycin A than any other leukemia cells tested, although 745A cells were not induced to differentiate by this antibiotic (data not shown).

Adriamycin, 1-β-D-arabinofuranosylcytosine, butyric acid, and hemin are potent inducers of erythroid differentiation of K562 cells (8, 9). Fig. 4 shows the effect of simultaneous treatment with herbimycin A and the other well-known inducers on induction of benzidine-positive cells after incubation for 4 days. At the concentrations used these inducers inhibited cell growth by 10-15%. The results show that herbimycin A and the other differentiation inducers have additive or more than additive effects on induction of benzidine-positive cells in suboptimal concentrations (Fig. 4).

Treatment with 4 x 10^{-8} M herbimycin A alone scarcely affected cell growth of K562 cells (Fig. 5), and the concentration of Adriamycin required for 50% inhibition of cell growth was 1.1 x 10^{-8} M. When K562 cells were treated simultaneously with 4 x 10^{-8} M herbimycin and 1.1 x 10^{-8} M Adriamycin, the cell number was decreased to less than 20% of that in control culture (Fig. 5). Similar results were obtained when the cells were treated with low concentrations of herbimycin and 1-β-D-arabinofuranosylcytosine. These results indicate that a low concentration of herbimycin A increases inhibition of cell growth of K562 cells by Adriamycin or 1-β-D-arabinofuranosylcytosine.

**DISCUSSION**

Herbimycin A has a variety of biological effects on a number of tumor cells, including reversion of transformed Rous sarcoma virus-infected rat kidney cells (12, 13, 22) to normal morphology, suppression of some tumor promotions induced by 12-O-tetradecanoylphorbol-13-acetate (23), and induction of erythroid differentiation.

Human K562 cells contain several tyrosine-phosphorylated proteins which were detected by antibody for phosphotyrosine. It has been demonstrated that p210_{c-abl} is an active tyrosine kinase and the major tyrosine-phosphorylated protein is p210_{c-abl} itself (5, 6). Thus, the tyrosine phosphorylation of these proteins is most likely due to the tyrosine kinase activity associated with p210_{c-abl}. Tyrosine phosphorylation of these proteins was reduced when K562 cells were induced to differentiate by hemin (11). The earliest reduction (approximately 30%) of tyrosine phosphorylation could be seen after a 6-h incubation with hemin. The reduction by herbimycin A was more rapid than that by hemin and the effective concentration was noncytotoxic. We suggest the possibility that protein phosphorylation may influence the differentiation of K562 cells, although other possibilities cannot be ruled out.

In a rat kidney cell line infected with Rous sarcoma virus, herbimycin A had no direct effect on tyrosine kinase, but it did destroy its intracellular environment, resulting in an irreversible alteration of the enzyme and loss of catalytic activity (22). The inactivation of tyrosine kinase by herbimycin in K562 cells is to be clear.

The antiproliferative effect of herbimycin A is different in each leukemia cell line. K562 cells are more sensitive to herbimycin among the human cell lines we tested. This suggests that tyrosine kinase activity may be critically involved in growth control mechanism of K562 cells, possibly as a result of induction of terminal differentiation. Friend virus-induced erythroleukemia cells are highly sensitive to herbimycin A in inhibition of cell growth. A type of tyrosine kinase might be involved in the mechanism of malignant transformation of Friend leukemia cells, although the precise mechanism of malignant transformation by Friend leukemia virus remains to be examined (24). Recently, Noguchi et al. (25) reported that herbimycin inhibited the action of erythropoietin on the proliferative response but not on the differentiation response of erythroid progenitor cells in mouse fetal liver. Herbimycin A and its derivatives might be useful as cancer chemotherapeutic agents against some types of leukemia oncogenesis where tyrosine kinase activities are implicated. Herbimycin A has target specificity but the effective concentration which inhibits tyrosine kinase activity derived from oncogenes is less cytotoxic in K562 cells. Since herbimycin A can have an additive or more than additive effect with some well-known antitumor agents such as Adriamycin or 1-β-D-arabinofuranosylcytosine these combinations may be useful for the treatment of some types of leukemia.

**REFERENCES**

DIFFERENTIATION OF K562 BY HERBIMYCIN

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