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 for erythroid differentiation by hemin, reduction in the intracellular level of tyrosine phosphorylation occurs. 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. Noncotoxic 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-B-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. Noncotoxic concentrations of herbimycin enhanced the antiproliferative effect of Adriamycin or 1-B-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 (p210<sup>°°</sup>) 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 p210<sup>°°</sup> 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 v-abl protein is known to be mediated by its tyrosine kinase activity. The tyrosine kinase activity of p210<sup>°°</sup> 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 p210<sup>°°</sup> 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

Received 5/24/98; revised 9/16/98; accepted 10/12/98.

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1 This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CML, chronic myelogenous leukemia; p210<sup>°°</sup>, structurally altered c-abl protein with a molecular weight of 210,000; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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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-1 (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% CO<sub>2</sub> 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 x 10<sup>4</sup>/ml) were suspended in 1 ml of culture medium and were cultured with or without herbimycin A at 37°C in a multidish (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<sup>i</sup> and Immunoprecipitation. Exponentially growing cells (2 x 10<sup>5</sup>/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 x 10<sup>-4</sup> M herbimycin A. Labeling was started by addition of 32P<sup>i</sup> (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 30 s at 2 x 10<sup>7</sup> cells/ml. The lysates were precipitated with agarose-linked protein A (Zymed, Los Angeles, CA). Immunoprecipitates were washed five times in 50 mM Tris-HCl (pH 7.5)-0.15 M NaCl 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 x 10<sup>-4</sup> M herbimycin A. Labeling was started by addition of 32P<sup>i</sup>, (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 30 s at 2 x 10<sup>7</sup> cells/ml in 50 mM Tris-HCl (pH 7.5)-0.15 M NaCl-0.1% SDS-1% Triton X-100-0.5% sodium deoxycholate-1 mM phenylmethylsulfonyl fluoride buffer. The cell extract was clarified by centrifugation at 16,000 x g for 10 min and the supernatant was diluted 10-fold with PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The diluted sample was treated with agarose-linked monoclonal antibody against phospho-tyrosine (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 phenylmethylsulfonyl fluoride 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.
DIFFERENTIATION OF K562 BY HERBIMYCIN

RESULTS

After incubation for 4 days herbimycin A was found to inhibit the growth of K562 cells at concentrations higher than 6 × 10⁻⁸ M, 50% inhibition of growth occurring at 9.5 × 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 × 10⁻⁷ M herbimycin A, which caused about 65% inhibition of cell growth.

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

<table>
<thead>
<tr>
<th>Herbimycin (× 10⁻⁸ M)</th>
<th>Cell no. benzidine-positive (x 10⁵/ml)</th>
<th>Benzidine-positive cell no. (x 10⁶/ml)</th>
<th>Hemoglobin (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.8</td>
<td>3.0</td>
<td>2.3</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>

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 × 10⁻⁸ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human K562 (erythroleukemia cells)</td>
<td>9.5 ± 0.3¹</td>
</tr>
<tr>
<td>HL-60 (promyelocytic cells)</td>
<td>103 ± 8</td>
</tr>
<tr>
<td>U937 (monocytic cells)</td>
<td>175 ± 9</td>
</tr>
<tr>
<td>M1 (myeloblastic leukemia cells)</td>
<td>14.4 ± 1.3</td>
</tr>
</tbody>
</table>

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 × 10⁻⁸ M herbimycin A. The cell extracts (3 × 10⁻⁷ cpm) were immunoprecipitated with phosphotyrosine monoclonal antibody (1) or anti c- abl 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.

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 × 10⁻⁴ M Adriamycin; B, 3.5 × 10⁻⁴ M 1-β-D-arabinofuranosylcytosine; C, 1 × 10⁻⁴ M hemin; D, 1 × 10⁻⁴ M butyric acid.

When the cells (5 × 10⁴/ml) treated with 7.5 × 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 \times 10^{-8} \text{M} \text{herbimycin and } 1.1 \times 10^{-8} \text{M} \text{Adriamycin}, the concentration of Adriamycin required for 50\% inhibition of cell growth was 1.1 \times 10^{-8} \text{M}. When K562 cells were treated simultaneously with 4 \times 10^{-8} \text{M} \text{herbimycin and } 1.1 \times 10^{-8} \text{M} \text{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-\beta-D-arabinofuranosylcytosine. These results indicate that a low concentration of herbimycin A increases inhibition of cell growth of K562 cells by Adriamycin or 1-\beta-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^{abl} is an active tyrosine kinase and the major tyrosine-phosphorylated protein is p210^{abl} itself (5, 6). Thus, the tyrosine phosphorylation of these proteins is most likely due to the tyrosine kinase activity associated with p210^{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-\beta-D-arabinofuranosylcytosine these combinations may be useful for the treatment of some types of leukemia.

**REFERENCES**

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