Herbimycin A, an Inhibitor of Tyrosine Kinase, Prolongs Survival of Mice Inoculated with Myeloid Leukemia C1 Cells with High Expression of v-abl Tyrosine Kinase

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ABSTRACT

Herbimycin A, a benzoquinonoid ansamycin antibiotic, reduces intracellular phosphorylation by some tyrosine kinases, including v-abl. The mouse megakaryoblastic cell line C1 expresses v-abl protein at high levels. Herbimycin A at about 20 ng/ml caused 50% inhibition of growth of C1 cells but at 100 ng/ml scarcely affected the growth of another mouse leukemia cell line, M1 cells, or of normal bone marrow cells. Injection of 10^6 C1 cells into nude mice resulted in death of all the mice within 30 days. Administration of herbimycin A significantly enhanced the survival of mice inoculated with C1 cells but scarcely affected the survival of mice inoculated with M1 cells. These results suggest that herbimycin A and/or related compounds may be useful for treatment of some types of leukemia in which tyrosine kinase activity is implicated as a determinant of the oncogenic state.

INTRODUCTION

The discovery of a large number of viral oncogene products with tyrosine kinase activity provided evidence for abnormal cell growth via tyrosine phosphorylation (1–3). Because the ability of these oncogenes to transform cells is dependent on the continued expression of tyrosine kinases (1, 4), the use of specific protein tyrosine kinase inhibitors is a rational approach in studies on the effects of oncogenic proteins on cellular transformation.

Chronic myelogenous leukemia and some cases of acute lymphocytic leukemia are characterized by the Philadelphia chromosome translocation, in which 5' sequences of the bcr gene become fused with the c-abl proto-oncogene. The resulting genes encode proteins with high activity as protein tyrosine kinases, and the oncogenic potentials of the bcr-abl fusion proteins have been demonstrated in vitro and also in mice (5–7). The transforming activity of the chimeric gene is closely associated with its tyrosine kinase activity. Therefore, a selective inhibitor of tyrosine kinase activity might be useful in chemotherapy of some leukemias with the Philadelphia chromosome (8).

Mouse C1 cells are megakaryoblastic cells established by coinfection with Abelson murine leukemia virus and recombinant SV40 (9). These cells are induced to differentiate into megakaryocytes by treatment with some inhibitors of tyrosine kinase, including herbimycin A, and inhibition of their v-abl tyrosine kinase activity is preceded by induction of their differentiation (10). Moreover, treatment of C1 cells with a v-abl antisense oligomer inhibits their proliferation and induces acetylcholinesterase activity, a typical marker of megakaryocytic differentiation (10). These findings suggest that a specific inhibitor of tyrosine kinase may also be an effective antitumor agent against various other types of tumors in which tyrosine kinase is implicated as a determinant of the oncogenic state.

Herbimycin A, isolated from the culture filtrate of Streptomyces sp. MH237-CF-8 (11), inhibits the activities of protein tyrosine kinases encoded by several oncogenes (12). The antibiotic was totally ineffective in reducing cyclic AMP-dependent protein kinase or protein kinase C but inactivated several protein tyrosine kinases, including v-src, v-abl, and bcr-abl kinases (10, 13). A noncytotoxic concentration of the antibiotic induced differentiation of K562 and KU812 cells, established from patients with Philadelphia chromosome-positive leukemia (14), and also mouse C1 cells (10). The marked in vitro effects of herbimycin A on growth and differentiation of C1 cells prompted us to examine the antileukemic effect of herbimycin A on mice inoculated with C1 cells, as an experimental model of leukemia with an oncogenic abl gene.

MATERIALS AND METHODS

Animals. Seven-week-old female athymic nude mice with a BALB/c genetic background were supplied by CLEA Japan (Tokyo, Japan). They were housed under specific pathogen-free conditions in Clean Racks (Sanki Kogyo, Tokyo, Japan).

Cells and Cell Culture. Mouse leukemia C1 and M1 cells (15) were cultured in suspension in Eagle's minimum essential medium with twice the normal concentrations of amino acids and vitamins, supplemented with 10% horse serum. PA6 cells (16, 17) were cultured with Eagle's minimum essential medium with twice the normal concentrations of amino acids and vitamins, supplemented with 10% horse serum, in collagen-coated dishes. Cells were subcultured by treatment with 0.05% trypsin-0.02% EDTA (GIBCO, Grand Island, NY) for 5 min, with vigorous shaking. The cells were cultured at 37°C in a humid atmosphere of 5% CO2 in air. C1 and/or bone marrow cells flushed from the femoral cavity were seeded onto confluent (7-day-old) PA6 cell layers. Twice a week, the medium, containing nonadherent cells, was removed by aspiration and replaced by fresh culture medium without herbimycin A.

Immunohistochemical Staining of SV40 T-Antigen. Cytocentrifuge preparations were fixed with acetone for 10 min and incubated with monoclonal antibody against SV40 T-antigen (Oncogene Science, Manhasset, NY). The immunocomplexes were detected using a biotin/avidin kit (Vector, Burlingame, CA).
Administration of Herbimycin A. Herbimycin A was a generous gift from Drs. S. Omura and Y. Uehara (Kitasato Institute and National Institute of Health, Tokyo, Japan). A stock solution (7.5 mg/ml) of this antibiotic was prepared in dimethyl sulfoxide. A stock solution of daunomycin (50 μg/ml) was prepared in phosphate-buffered saline. Mice were inoculated i.p. with 1 x 10⁶ of either stock solution, with the first injection being given 24 h after inoculation of leukemia cells.

RESULTS

Selective Growth-inhibitory Effect of Herbimycin A on C1 Cells. C1 cells expressed a high level of protein tyrosine kinase activity (10), while M1 cells, another leukemia cell line, did not. The myeloid leukemia M1 cells, derived from a spontaneous myeloid leukemic mouse, expressed high levels of c-myc and c-myb proto-oncogenes (19). The elevated expression of myc and myb might be responsible for maintaining M1 cells at the blastic stage. Herbimycin A inhibited growth of C1 cells more than that of another leukemia cell line, M1 cells. Treatment with 100 ng/ml herbimycin A scarcely affected the cell growth of M1 cells, but herbimycin A at about 20 ng/ml for 5 days caused 50% inhibition of growth of C1 cells (Fig. 1). The growth-inhibitory effect was cytostatic rather than cytotoxic; the number of C1 cells increased slightly even at a high concentration of herbimycin A, with >90% of the treated cells remaining viable. Herbimycin A induced differentiation of C1 cells, but not M1 cells, into megakaryocytes (data not shown) (8, 10).

The mouse preadipose cell line PA6 can support proliferation of normal hematopoietic stem cells, defined as CFU-S, and sustain hematopoiesis for several weeks (16, 17). The growth of C1 cells on a PA6 cell layer was similar to that in the absence of the PA6 cell monolayer, and the inhibitory effect of herbimycin A was not significantly affected by the presence of PA6 cells (Fig. 2). On the other hand, the proliferation of normal hematopoietic stem cells was not significantly affected by even a high concentration of 50 ng/ml herbimycin A (Table 1).

Moreover, maturation of normal hematopoietic stem cells was scarcely affected by treatment with herbimycin A, since many myeloid cells (neutrophils, monocytes, mast cells, and megakaryocytes) at various differentiation stages were observed in herbimycin A-treated cultures, as in untreated cultures.

The results of a typical experiment, in which 1 x 10⁶ bone marrow cells plus 0.5 x 10⁵ C1 cells were cultured in the presence of confluent PA6 cell layers, are shown in Fig. 2. When co-cultured on a monolayer of PA6 cells in the absence of herbimycin A for 7 days, growth of C1 cells predominated over that of normal hematopoietic stem cells. Destruction of the PA6 cell layer in all regions of the dish was observed after 10 days (data not shown). Most of the normal progenitor cells were round and uniform in size, whereas most of the C1 cells formed aggregates of irregular sizes and single C1 cells were spindle-shaped.

C1 cells were easily distinguished from normal progenitor cells by their sizes and megakaryoblastic morphology (Fig. 3, left). This distinction was confirmed immunochemically with antibody against T-antigen of SV40 (Fig. 3, right), inasmuch as C1 cells express a high level of T-antigen of SV40 (9). More than 70% of the nonadherent cells in 7-day cultures were C1 cells. Herbimycin A inhibited cell proliferation and induced megakaryocytic differentiation of C1 cells in co-cultures with normal bone marrow cells, as in cultures of C1 cells alone. Differentiated C1 cells were observed among nonadherent cells in co-cultures treated with herbimycin A for 4 days, and the differentiated C1 cells still expressed a high level of T-antigen. Treatment with herbimycin A greatly reduced the proliferation of C1 cells but not of normal hematopoietic cells, which differentiated into neutrophils, monocytes, mast cells, and megakaryocytes. After co-culture with >50 ng/ml herbimycin A for 1 week, <5% of the nonadherent cells appeared to be C1 cells, morphologically or immunochemically (Figs. 2 and 3). When all the cells were harvested after co-culture for 2 weeks
Table 2 Effect of herbimycin A on survival times of mice inoculated with leukemia cells

<table>
<thead>
<tr>
<th>Leukemia cells</th>
<th>Treatment (μg/mouse)</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Control</td>
<td>24.1 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Herbimycin A 7.5</td>
<td>27.7 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>41.3 ± 12.0a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>35.9 ± 7.3</td>
</tr>
<tr>
<td></td>
<td>Daunomycin 5</td>
<td>43.1 ± 10.4a</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40.8 ± 9.1a</td>
</tr>
<tr>
<td>M1</td>
<td>Control</td>
<td>35.9 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>Herbimycin A 15</td>
<td>34.6 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Daunomycin, 5</td>
<td>61.6 ± 4.4a</td>
</tr>
</tbody>
</table>

*P < 0.01, by Student's t test.

and PA6 cells were separated from C1 cells and normal hematopoietic cells, no C1 cells (<0.1% of the total cells) were detectable immunochemically. These results suggest that herbimycin A selectively inhibited the proliferation of C1 cells.

Effect of Herbimycin A on the Survival Time of Mice Inoculated with C1 Cells. After inoculation of 10⁶ C1 cells, all the mice died of frank leukemia within 30 days. Bone marrow smears showed that C1 cells had infiltrated into the bone marrow 21 days after their inoculation. Most of the leukemic mice showed moderate enlargement of the spleen and peripheral lymph nodes. No other organ showed infiltration by the blastic cells. To test the effect of herbimycin A on the leukemogenicity of C1 cells, we administered graded amounts of herbimycin A i.p. to mice inoculated with C1 cells (Table 2). Fig. 4 shows that the mean survival time of untreated mice inoculated with C1 cells was about 23 days, whereas that of mice treated with 15 μg of herbimycin A was about 34 days. Daunomycin, a representative antileukemic drug, also prolonged the survival of mice inoculated with C1 cells. Treatment with herbimycin A was as effective as that with daunomycin (Table 2). Daily injections of 15 μg of herbimycin A did not prolong the survival of mice inoculated with 10⁶ M1 cells (Table 2). These in vivo findings on the proliferation of survival of mice are consistent with in vitro findings on growth inhibition by herbimycin A (Fig. 1).

DISCUSSION

The preadipose cell line PA6 can support proliferation of CFU-S only when PA6 cells are in contact with the stem cells, and the entire process of hematopoiesis takes place in intimate association with the preadipocyte layers (16, 17). PA6 cells do not synthesize a detectable level of mRNA for several hematopoietic growth factors. The present results show that herbimycin A does not significantly affect the hematopoiesis supported by stroma cells through direct cell-to-cell interaction. However, herbimycin A significantly inhibited colony formation of normal bone marrow cells stimulated by some hematopoietic growth factors, such as granulocyte-macrophage colony-stimulating factor and interleukin 3 (data not shown). The growth factors appear to use a tyrosine kinase-dependent pathway to mediate their biological effects. This may explain, at least in part, the difference in their sensitivities to herbimycin A.

The mouse M1 cell line is a good experimental model for use in studies on differentiation therapy (20, 21). Several differentiation inducers significantly enhance the survival of mice inoculated with M1 cells (20), and the effects of these inducers are consistent with their effects on differentiation of the cells in vitro and in vivo, suggesting that prolongation of the survival of mice may be associated with stimulation of differentiation of the cells by the inducers. Herbimycin A does not induce differentiation of M1 cells or inhibit their growth, but it induces differentiation of C1 cells. Its differentiation-inducing and growth-inhibitory effects are compatible with its effects in prolonging survival of mice inoculated with leukemia cells.

C1 cells had infiltrated into the bone marrow 21 days after their inoculation. However, in approximately 70% of the mice inoculated with 10⁶ C1 cells, solid tumors developed at the s.c. sites of leukemia cell injection. The survival of mice without solid tumors was greatly enhanced by treatment with herbimycin A, but the formation of solid tumors reduced its therapeutic
effect. The sizes of s.c. tumors varied for the tumor-bearing mice, and they were roughly antiparallel to the therapeutic effect. This was also true in the case with daunomycin, a typical antileukemic agent. Thus, the therapeutic effect of herbimycin A was not as great as expected from its in vitro effect but was comparable to that of daunomycin.

Herbimycin A enhanced the survival of immunodeficient (athymic nude) mice inoculated with C1 cells, suggesting that T-lymphocyte-mediated immune mechanisms may not be directly involved in its therapeutic effect. It is also unlikely that its prolongation of survival was mainly due to a host-mediated effect, because it did not affect the survival of mice inoculated with M1 cells.

Autologous bone marrow transplantation has the potential for cure of leukemia if residual clonogenic leukemia cells in the graft can be eliminated or reduced in number. Various techniques have been developed to eliminate malignant cells in vitro (22–27). The present results suggest that herbimycin A and related compounds may be very effective for eliminating malignant cells from the bone marrow of patients with leukemias in which tyrosine kinase activity is implicated as a determinant of the oncogenic state. Treatment with a specific tyrosine kinase inhibitor appears to be a promising method for improving the therapeutic efficacy of autologous bone marrow transplantation.

We found previously that the growth-inhibitory and differentiation-inducing effects of herbimycin A differed in different human leukemia cells; Philadelphia chromosome-positive (or bcr-abl gene-containing) leukemia cells appeared to be more sensitive to herbimycin A than Philadelphia chromosome-negative cells (14). Further studies on the effects of herbimycin A and related compounds will contribute to the development of new strategies for treatment of Philadelphia chromosome-positive leukemia.

REFERENCES

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