Reversal of Transformed Phenotypes by Herbimycin A in src Oncogene Expressed Rat Fibroblasts

Yuko Murakami, Satoshi Mizuno, Makoto Hori, and Yoshimasa Uehara

National Institute of Health, Department of Antibiotics, 2-10-35 Kismokosai, Shinagawa-ku, Tokyo 141, Japan [Y. M., S. M., Y. U.]; and Showa College of Pharmaceutical Science, 5-1-8 Tsurumaki, Setagaya-ku, Tokyo 154, Japan [M. H.]

ABSTRACT

We studied the effectiveness of herbimycin A, an inhibitor of the function of the src oncogene, to reverse the various transformed phenotypes in normal rat kidney (NRK) cells integrating temperature-sensitive src (ts/NRK). Elevated glucose transport in ts/NRK cells at a permissive temperature (33°C) was decreased by herbimycin in 8 h to near the level that was observed either in ts/NRK cells grown at a nonpermissive temperature (39°C) or in untransformed NRK cells at either temperature. Herbimycin caused no significant decrease in glucose uptake in ts/NRK cells grown at 39°C. The effects of herbimycin on serum- and anchorage-independent growth properties of ts/NRK cells and of NRK cells integrating K-ras (KNRK) were also examined. With ts/NRK cells grown at 33°C, the inhibition of cell growth by herbimycin became more pronounced when the serum concentration in the medium was lowered. With KNRK cells, in contrast, almost the same extent of cell growth inhibition was exerted by herbimycin irrespective of the serum concentration. Furthermore, with ts/NRK cells grown at 33°C, herbimycin inhibited the colony formation in the soft agar medium more strongly than on a solid support. No such differential effects were observed with KNRK cells under similar conditions. These results suggest that herbimycin specifically acts on cells expressing the src oncogene and reverses various transformed characteristics to the normal ones.

INTRODUCTION

Evidence has been accumulating that the malfunctioning of cellular oncogenes is a cause of human cancers (1, 2). Therefore, a compound which inhibits the activity of an oncogene product may provide a new means to overcome some tumors. We previously isolated a benzoquinonoid ansamycin antibiotic, herbimycin, as an active substance that caused morphological changes, and growth in soft agar medium (for a review see Ref. 5). We observed previously that cytoplasmic organization of actin (actin fiber) was rebuilt by the treatment of herbimycin (4). More recently Umezawa et al. reported the enhanced gene expression for a cell surface glycoprotein, fibronectin, in herbimycin-treated ts/NRK cells (6).

Although it is clear that continuous expression of the src gene, i.e., continuous presence of protein kinase activity of p60Src, is required for maintenance of these parameters of transformation (7, 8), some of these parameters have been reported to be dissociated in cells infected with various temperature-dependent mutants of RSV (9). Therefore, it seemed important to test whether herbimycin changes, even if reversibly (4), other transformed phenotypes such as the elevated level of glucose transport, the lowered serum requirement and anchorage independence for cell growth, in order to test the application of such inhibitors as cancer chemotherapeutic agents.

MATERIALS AND METHODS

Cells and Culture Conditions. A rat kidney cell line (NRK) infected with ts25, a T-class mutant of Rous sarcoma virus Prague strain (10), was provided from M. Yoshida, Cancer Institute, Tokyo, Japan, and is abbreviated as ts/NRK. The cells were cultured at 33°C (a permissive temperature) or at 39°C (a nonpermissive temperature) in Dulbecco's modified Eagle medium (GIBCO Laboratories, Grand Island, NY) supplemented with, unless otherwise indicated, 10% of heat-inactivated calf serum in humidified air and 5% CO2. KNRK and uninfected NRK-49F cells were obtained from American Type Culture Collection, Rockville, MD.

To examine the serum dependency of cell growth, cells were seeded initially at a density of 5 × 104 per 35-mm dish in the medium containing 5% calf serum. Next day the medium was replaced by a fresh medium containing either 5, 1, or 0.2% of calf serum after washing with respective medium.

Measurement of 2-Deoxyglucose Uptake. For glucose transport studies, cells (corresponding to 100–200 μg proteins) cultured on 35-mm dishes were washed three times with Dulbecco's PBS, and then incubated for 10 min at room temperature in PBS containing 2-deoxy-d-[3H]glucose (0.4 μCi/ml) (Amersham International plc) with 1 μM unlabeled 2-deoxy-d-glucose. Uptake was stopped by washing cells five times with cold PBS and the cells were scraped off in 1.0 ml water with a rubber policeman. A portion of cell lysates was used for protein determination by the method of Lowry et al. (11), and the rest for radioactivity measurement in a scintillation counter. Uptake was linear with labeling time of the first 20 min or longer.

Colony Formation. For the soft agar cloning, the layers of exponentially growing cells were washed twice with PBS, treated with trypsin, and squirted with a syringe (26 gauge) into the medium containing 10% serum. After the dishes were incubated for 2 weeks, the colonies >50 cells were stained with 0.1% crystal violet. The colonies consisting of >50 cells in diameter were counted.

For the liquid cloning, 2-ml aliquots of cell suspension (100 cells/ml) were added to culture dishes, each had contained 4 ml of prewarmed (33°C or 39°C) medium. The dishes were then incubated for 2 weeks without changing the medium at respective temperatures. Colonies were washed free of the medium, fixed with 4% formalin in PBS, and stained with 0.1% crystal violet. The colonies consisting of >50 cells were counted.

RESULTS

Effect of Herbimycin on Glucose Uptake in ts/NRK Cells. The initial rate of 2-deoxy glucose uptake was measured in ts-RSV-
infected NRK (ts/NRK) cells. As shown in Table 1, ts/NRK-cells cultured at a permissive temperature (33°C) had a 2- to 3-fold increase in the rate of sugar uptake than the cells cultured at a nonpermissive temperature (39°C). The rate of glucose uptake into uninfected NRK cells at either temperature was almost the same as that of ts/NRK cells grown at 39°C, indicating that the elevated glucose transport is a transformed phenotype induced by viral src oncogene.

First we measured the time course of the changes in glucose uptake during the herbimycin treatment and the result was compared with that observed following a shift in temperature from 33°C to 39°C. As shown in Fig. 1, the rate of sugar uptake declined in the first 8 h of the treatment with herbimycin, and remained almost constant thereafter. On the other hand, the decline of glucose uptake following the temperature shift occurred more slowly and continued for 24 h.

Next we treated ts/NRK cells with various concentrations of herbimycin for 21 h at either temperature. The rate of glucose uptake of the cells cultured at 33°C declined to near the level of normal state cells (39°C) which was little affected by herbimycin (Fig. 2).

Effect of Herbimycin on the Serum Requirement for Growth of Transformed Cells. Fig. 3 shows the serum requirement for growth of ts/NRK cells at 33°C and 39°C. A reduced requirement of transformed cells (at 33°C) compared with that of normal cells (at 39°C) was apparently demonstrated. As shown in Fig. 4 (top), the growth inhibition by herbimycin of ts/NRK cells at 33°C was more pronounced at lower concentrations of serum. For comparison, we conducted a parallel experiment with KNRK cells which is transformed by viral K-ras oncogene. With this cell line the degree of inhibition by herbimycin did not change greatly at different serum concentrations (Fig. 4, bottom).

Table 1 Comparison of rate of glucose uptake at 33°C and 39°C in ts/NRK and uninfected NRK cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell line</th>
<th>Growth temperature (°C)</th>
<th>2-Deoxy-[3H]glucose uptake (cpm/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ts/NRK</td>
<td>33</td>
<td>31.0</td>
</tr>
<tr>
<td>2</td>
<td>ts/NRK</td>
<td>39</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>ts/NRK</td>
<td>33</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>NRK-49F</td>
<td>33</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>10.9</td>
<td></td>
</tr>
</tbody>
</table>

*Values, averages of two determinations with the maximum range of 10%. Measurement of glucose uptake is described in “Materials and Methods.”
**Effect of Herbimycin on Anchorage Independent Cell Growth.** When ts/NRK cells were incubated at 33°C, they formed colonies in soft agar medium (anchorage-independent growth) as well as on a solid support (anchorage-dependent growth) but when cultured at 39°C they could only proliferate on a solid support (Fig. 5). Does herbimycin selectively inhibit anchorage-independent growth? To answer this, we first examined the effect of herbimycin on the colony formation of ts/NRK cells in soft agar at 33°C. Exposure of the transformed cells to various concentrations of herbimycin for 2 weeks at 33°C in soft agar caused a decrease in the number of colonies dose dependently, and few colonies were formed at 0.5 μg/ml. In order to distinguish whether this is due to reversion of the transformed phenotype of the cells to the normal one or simply due to the cytotoxicity of herbimycin, a parallel experiment determining colony formation on a solid support was conducted for comparison. At 0.5 μg/ml of herbimycin, the number of colonies formed was about 50% of control and at a wide range of herbimycin concentrations significantly greater numbers of colonies were formed on a solid support (Figs. 5 and 6).

To exclude the possibility that the different effects of herbimycin on the colony forming ability in the two assay systems is simply due to the difference in the assay methods, we further conducted a similar experiment using KNRK cells and the result was compared with that with ts/NRK cells. As shown in Fig. 7, herbimycin exhibited no differential inhibitory effect on the colony formation of KNRK cells in the two assays, strongly suggesting that herbimycin did inhibit anchorage-independent growth of ts/NRK cells but not of KNRK cells.

**DISCUSSION**

The inhibitory effect of herbimycin on the function of src oncogene was initially noticed by the alteration of the transformed morphologies of ts/NRK cells to the normal ones at 33°C (3). In the present paper, we demonstrated that several other transformed phenotypes were also reversed by herbimycin to the normal phenotypes.

Elevated glucose transport is a well-established biochemical feature of virally transformed cells (12). Transformation increases the number of glucose transport proteins in the plasma membrane (13–15). Herbimycin significantly, although incompletely, reduced the elevated glucose uptake of ts/NRK cells at 33°C to the normal level of untransformed NRK cells, with little effect on the glucose transport of the cells grown at 39°C where the src gene product is not functioning. These results imply that the reduction of glucose uptake is the result of the inhibition by herbimycin of the src gene product and that herbimycin has no effect on the glucose transporter per se. Although herbimycin was two times more cytotoxic to 39°C cells than against 33°C cells in a solid support cloning assay (not shown), glucose transport was not inhibited in 39°C cells (Fig. 2). This indicates that the inhibition of transformed phenotypes is differentiated from the cytotoxic effects of the antibiotic.

A lowered requirement for growth factors and a lowered anchorage dependence of cell growth are the characteristic properties of tumor virus transformed cells (16, 17). Concerning the requirement for growth factors, the growth inhibition by herbimycin of src-transformed cells (ts/NRK at 33°C) became more pronounced when the serum concentration in the medium was lowered. It was inappropriate to examine the growth inhibition by herbimycin at 39°C because the increase in cell numbers was too small at low serum concentrations (Fig. 3). It is obvious, however, that src-transformed cells but not ras-transformed cells acquired serum dependency when exposed to herbimycin.

Among the various characteristics commonly associated with the transformed cell phenotype, growth in the absence of a solid support (anchorage independency) was found to be well correlated with in vivo tumorigenicity (17). Therefore, it was important to know whether herbimycin interferes with colony formation in src-transformed cells in soft agar, especially in connection with possible use in chemotherapy. ts/NRK cells, when grown at 39°C, formed no visible colonies in soft agar and the seeded single cells remained as single cells during the incubation, in contrast to active colony formation at 33°C. This indicates that the anchorage-independent cell growth phenotype is strictly controlled by active src gene product (expressed at 33°C but not at 39°C) in this cell line. The effect of herbimycin...
on this phenotype appeared somewhat leaky because we observed some small colonies in the soft agar medium (less than 0.5 mm in diameter, uncounted as a colony) besides single cells in the herbimycin-treated culture at 33°C. This may be due to incomplete inhibition of src function by herbimycin or induction of some herbimycin-resistant clones during the culture with herbimycin.

The biochemical basis for the inhibition of various transformed phenotypes should be due to the inhibition of intracellular p60src kinase activity (4) which is reflected by the reduction of total cellular phosphotyrosine level in the cells.4 Herbimycin was also found effective in inhibiting transforming activity of other tyrosine kinase family oncogenes (yes, fps, ros etc.) in various types of cells including chicken, mouse and vole (18).

Recent studies show that herbimycin and related antibiotics and analogs have some antitumor activities against Ehrlich ascites carcinoma, P388, and Lewis lung carcinoma in vivo (19–21). However, it is not clear whether the chemotherapeutic activities of the herbimycin family in these animal tumor model systems are related to the inhibitory activity of src or src-related gene function.

REFERENCES


diagram

Fig. 6. Effect of herbimycin on the formation of colonies on a solid support and in soft agar. Numbers of colonies formed on a solid support or in soft agar in the presence of various concentrations of herbimycin were counted after 14 days of incubation at 33°C. Two separate experimental data are plotted by different symbols [experiment 1 (○, O), experiment 2 (△, △)]; points, mean of duplicate or triplicate dishes.

Fig. 7. Effects of herbimycin on colony formation of src or ras oncogene-expressed NRK cells. Numbers of colonies of KNRK cells were counted after 10 days culture at 37°C. For ts/NRK cells, the data of experiment 1 shown in Fig. 6 were used. Bars, averages of three plates; thin lines at top of bars, SD.

*Manuscript in preparation.
Reversal of Transformed Phenotypes by Herbimycin A in src Oncogene Expressed Rat Fibroblasts

Yuko Murakami, Satoshi Mizuno, Makoto Hori, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/6/1587

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.