Control of Proliferating Potential of Myeloid Leukemia Cells during Long-Term Treatment with Vitamin D<sub>3</sub> Analogues and Other Differentiation Inducers in Combination with Antileukemic Drugs: In Vitro and in Vivo Studies<sup>1</sup>

Takashi Kasukabe,<sup>2</sup> Yoshiro Honma, Motoo Hozumi, Tatsuo Suda, and Yasuho Nishii

Department of Chemotherapy, Saitama Cancer Center Research Institute, Ina-machi, Kitada-ku, Saitama 362 [T. K., Y. H., M. H.]; Department of Biochemistry, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142 [T. S.]; and Research Laboratories of Chugai Pharmaceutical Company, Takada, Toshima-ku, Tokyo 171 [Y. N.], Japan

ABSTRACT

Growth inhibition of murine and human myeloid leukemia cells by differentiation inducers during long-term culture was examined to improve the strategy for therapy of myeloid leukemia by differentiation inducers. When the effect of 1α,25-dihydroxyvitamin D<sub>3</sub>, a typical differentiation inducer, on proliferation of mouse myeloid leukemia M1 cells was examined at a constant product of time and concentration (480 nM in 20 days), the continuous treatment with 24 nM 1α,25-dihydroxyvitamin D<sub>3</sub> was the most effective for inhibition of cell proliferation. After 20 days, the cumulative cell number was reduced about 3 x 10<sup>6</sup> times by continuous treatment with 24 nM 1α,25-dihydroxyvitamin D<sub>3</sub>. Similar results were obtained when M1 cells were treated continuously with dexamethasone.

M1 cells resistant to 1α,25-dihydroxyvitamin D<sub>3</sub> appeared about 25 days after the start of continuous treatment with 24 nM 1α,25-dihydroxyvitamin D<sub>3</sub>. On the other hand, when M1 cells were treated continuously with 1α,25-dihydroxyvitamin D<sub>3</sub> and noncytotoxic doses of antileukemic drugs such as 1-ß-D-arabinofuranosylcytosine and daunomycin, resistant cells did not appear for at least 35 days. A similar effect of 1α,25-dihydroxyvitamin D<sub>3</sub> and antileukemic drugs on cell proliferation was observed with the human monoblast-like cell line U937. The survival of syngeneic SL mice inoculated with M1 cells was prolonged more by treatment with both 1α-hydroxyvitamin D<sub>3</sub> and daunomycin than by treatment with either drug alone. These results suggest that continuous treatment with both differentiation inducers and certain antileukemic drugs may be more effective therapeutically than treatment with a differentiation inducer alone alone.

INTRODUCTION

Several myeloid leukemia cell lines have been induced to differentiate into mature granulocytes and macrophages by treatment with various compounds (1-7). Differentiation is associated with loss of mitotic activity. Mouse myeloid leukemia M1 cells, established from a spontaneous leukemia in an SL strain mouse, have leukemogenic activity in syngeneic mice (8). The cells were induced to differentiate in vitro and in vivo by various inducers, such as 1α,25(OH)<sub>2</sub>D<sub>3</sub> and dexamethasone. Moreover, inducers of cell differentiation significantly enhanced the survival times of mice inoculated with M1 cells (2, 9, 10). These results may be significant for leukemia therapy.

Most inducer-treated M1 cells were committed to differentiate into nondividing mature cells, but it was difficult to induce 100% of the cell population to differentiate. Many differentiating leukemia cell lines tend to become resistant to inducers of cell differentiation when they are cultured with some inducers or drugs for a long time (2, 11-14), and if any leukemic cells survive, the disease cannot be cured. Therefore, compounds must be found that induce 100% differentiation of the cells.

There are many reports about inhibition of cell growth of myeloid leukemia cells by differentiation inducers in short-term culture. Expressions of Fc and C3 receptors, phagocytosis, and lysosome appeared within 3 days after treatment of M1 cells with differentiation inducers such as 1α,25(OH)<sub>2</sub>D<sub>3</sub> (15). Morphological changes and decrease in cell proliferation also became significant after these treatments. However, little is known about the long-term effects of differentiation inducers on cell proliferation. In the present work, we tested the effects of differentiation inducers on proliferation of myeloid leukemia cells in long-term cultures to develop a more effective therapeutic strategy using differentiation inducers. We found a striking effect of 1α,25(OH)<sub>2</sub>D<sub>3</sub> and a noncytotoxic dose of antileukemic drug in inhibiting cell proliferation and inducing differentiation of myeloid leukemia cells. Then, on the basis of our in vitro data, we examined the effect of 1α(OH)D<sub>3</sub> plus daunomycin on the survival of mice inoculated with M1 cells.

MATERIALS AND METHODS

Cells and Cell Cultures. The cell lines used were myeloid leukemia M1 cells that were established from an SL mouse with myeloid leukemia (8). M1-LS cells were 1α,25(OH)<sub>2</sub>D<sub>3</sub>-less sensitive M1 subclone cells isolated spontaneously from parent M1 cells. Only 30-50% of M1-LS cells were induced to express phagocytic activity, a differentiation marker of M1 cells, even with more than 24 nM 1α,25(OH)<sub>2</sub>D<sub>3</sub> for 3 days. The U937 cell line was derived from a pleural effusion of a patient with histiocytic lymphoma and was shown to possess monoblast-like characteristics (16). M1 cells were maintained in Eagle's minimum essential medium with 2-fold the normal concentrations of amino acids and vitamins and supplemented with 10% heat-inactivated calf serum. U937 cells were maintained in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum. The cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

Inducers of Cell Differentiation. 1α,25(OH)<sub>2</sub>D<sub>3</sub> and 1α(OH)D<sub>3</sub> were obtained from Chugai Pharmaceutical Co., Tokyo, Japan. Dexamethasone was purchased from Sigma Chemical Co., St. Louis, MO. Conditioned medium from the peritoneum of syngeneic SL mice was used as an endogenous proteinous inducer for differentiation of M1 cells and was prepared as follows. Skin flaps in the abdominal region were deflected, and the abdominal wall was excised as one layer and washed 3 times with 20 ml of sterilized PBS. The washed peritoneal wall was cultured in 10 ml of culture medium for 3 days. The conditioned medium was collected by centrifugation, filtered through an HA 0.4-µm pore size filter (Millipore Corp., Bedford, MA) and stocked at -20°C until use.

Antileukemic Drugs. ara-C, daunomycin, and actinomycin D were obtained from Sigma.

Assay of the Properties of Differentiated Cells. For assay of phagocytic activity, cells were incubated for 4 h with a suspension of polystyrene latex particles (9). Then the cells were washed 3 times and the number of phagocytic cells among at least 300 viable cells was counted. Cells with more than 5 particles were counted as phagocytic cells.
Assay of the Cumulative Cell Number. The cell density of untreated cells was kept at $1 \times 10^4$–$2 \times 10^5$/ml to maintain continuous logarithmic growth. The cell density of the inducer-treated cells was kept at $2 \times 10^5$–$8 \times 10^5$/ml to maintain growing phase. The medium of treated cultures was replaced by fresh medium with or without differentiation inducers at least every 3 days to remove cell debris of terminally differentiated cells that died. The cell number was counted with an automatic cell counter (Coulter Counter, Model ZBI; Coulter Electronics, Inc., Hialeah, FL). The cumulative cell number was calculated from the counts and the dilution used when feeding the culture. This prolonged treatment with differentiation inducers and/or antileukemic drugs was not significantly cytotoxic to the culture cells, and the viability of the treated leukemic cells was more than 80% as assessed by trypan blue dye exclusion test.

Animals. Inbred SL strain mice were maintained as previously reported and were used at 8–10 weeks old (about 20 g; range: 18–22 g) for experiments (17).

Administration of 1α(OH)D$_3$ and Daunomycin. A stock solution (250 nmol/ml) of 1α(OH)D$_3$ was prepared in absolute ethanol. A stock solution (88.5 nmol/ml) of daunomycin was prepared in PBS. Mice were inoculated i.p. with $3 \times 10^5$ M1 cells and then treated i.p. 3 times a week with 0.2 ml of PBS or daunomycin stock solution containing 0.2 μl of 1α(OH)D$_3$ stock solution. The first injection of 1α(OH)D$_3$ and/or daunomycin was given 24 h after tumor challenge.

RESULTS

Effect of 1α,25(OH)$_2$D$_3$ on Proliferation of M1 Cells. To obtain a more effective protocol for treatment with 1α,25(OH)$_2$D$_3$, we examined the effect of 1α,25(OH)$_2$D$_3$ on proliferation of M1 cells at a constant product of time and concentration (480 nm/day) (i.e., at 24 nm continuously for 20 days, at 48 nm for 10 days from days 0–10, or from days 0–5 and from days 10–15, at 96 nm for 5 days from days 0–5, at 240 nm for 2 days from days 0–2, and at 480 nm for 1 day from day 0–1. Fig. 1 shows the time courses of cell proliferation of M1 cells after these various treatments with 1α,25(OH)$_2$D$_3$. Continuous treatment with 24 nm 1α,25(OH)$_2$D$_3$ for 20 days was the most effective for inhibiting cell proliferation: the cell number increased until day 5, but remained almost unchanged thereafter.

When M1 cells were treated with 1α,25(OH)$_2$D$_3$ at 48 nm for 10 days from days 0–10 and then cultured without 1α,25(OH)$_2$D$_3$, growth of M1 cells stopped from days 5–10, but started again when the M1 cells were cultured without 1α,25(OH)$_2$D$_3$ and thereafter was similar to that of untreated M1 cells. It is noteworthy that treatment with 48 nm 1α,25(OH)$_2$D$_3$ for a total of 10 days from days 0–5 and from days 10–15 was much less effective than continuous treatment at 48 nm for 10 days. These results indicate that more than 5 days of continuous treatment with 48 nm 1α,25(OH)$_2$D$_3$ is necessary to obtain significant growth inhibition of M1 cells.

After 20 days of exposure, the cumulative cell number was reduced $3 \times 10^2$ times by continuous treatment with 24 nm of 1α,25(OH)$_2$D$_3$ (Table 1). Similar results were obtained when the product of time and concentration of 1α,25(OH)$_2$D$_3$ was varied (Table 1). These results indicate that continuous treatment with 1α,25(OH)$_2$D$_3$ even at low concentration was the most effective for inhibiting M1 cell proliferation.

Effects of Dexamethasone and Proteinous Inducers on Proliferation of M1 Cells. Most M1 cells treated with dexamethasone were committed to differentiate within 3 days (2). When cells were cultured with various concentrations of dexamethasone for 3 days, and then washed and cultured without the inducer, cells treated with less than 1 x $10^{-5}$ M dexamethasone did not show complete arrest of proliferation. Those treated with 4 x $10^{-5}$ M dexamethasone did show complete arrest of proliferation (Fig. 2), but this concentration is much higher than the physiological concentration of glucocorticoids. Therefore, the

Table 1 Effects of various treatments with 1α,25(OH)$_2$D$_3$ on growth of M1 cells

<table>
<thead>
<tr>
<th>Concentration of 1α,25(OH)$_2$D$_3$ (nm)</th>
<th>Days of treatment*</th>
<th>Product of time and concentration (nm × day)</th>
<th>Cumulative cell no./ml at day 20, mean ± SD (×10$^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>44,800 ± 2,800</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>0–20</td>
<td>48</td>
<td>29.7 ± 1.4</td>
</tr>
<tr>
<td>4.8</td>
<td>0–10</td>
<td>48</td>
<td>59.3 ± 5.6</td>
</tr>
<tr>
<td>9.6</td>
<td>0–5</td>
<td>48</td>
<td>10,400 ± 17</td>
</tr>
<tr>
<td>4.8</td>
<td>0–5, 10–15</td>
<td>48</td>
<td>7,270 ± 230</td>
</tr>
<tr>
<td>24</td>
<td>0–2</td>
<td>48</td>
<td>26,500 ± 210</td>
</tr>
<tr>
<td>48</td>
<td>0–1</td>
<td>48</td>
<td>21,500 ± 200</td>
</tr>
<tr>
<td>24</td>
<td>0–20</td>
<td>480</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>48</td>
<td>0–10</td>
<td>480</td>
<td>9.8 ± 0.48</td>
</tr>
<tr>
<td>96</td>
<td>0–5</td>
<td>480</td>
<td>6,140 ± 360</td>
</tr>
<tr>
<td>48</td>
<td>0–5, 10–15</td>
<td>480</td>
<td>2,600 ± 100</td>
</tr>
<tr>
<td>240</td>
<td>0–2</td>
<td>480</td>
<td>18,700 ± 50</td>
</tr>
<tr>
<td>480</td>
<td>0–1</td>
<td>480</td>
<td>17,400 ± 80</td>
</tr>
<tr>
<td>240</td>
<td>0–20</td>
<td>4,800</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>480</td>
<td>0–10</td>
<td>4,800</td>
<td>17.3 ± 0.0</td>
</tr>
<tr>
<td>960</td>
<td>0–5</td>
<td>4,800</td>
<td>6,920 ± 60</td>
</tr>
<tr>
<td>480</td>
<td>0–5, 10–15</td>
<td>4,800</td>
<td>1,470 ± 170</td>
</tr>
<tr>
<td>2,400</td>
<td>0–2</td>
<td>4,800</td>
<td>10,000 ± 200</td>
</tr>
<tr>
<td>4,800</td>
<td>0–1</td>
<td>4,800</td>
<td>9,260 ± 650</td>
</tr>
</tbody>
</table>

* The culture medium was replaced by fresh medium at least once every 3 days. The cell density of the treated cells was kept at $2 \times 10^5$–$8 \times 10^5$/ml.
GROWTH CONTROL BY DIFFERENTIATION INDUCERS

Fig. 2. Proliferation of M1 cells in long-term culture with dexamethasone. M1 cells were cultured with $1 \times 10^{-7}$ M dexamethasone continuously (○); with $1 \times 10^{-6}$ M dexamethasone for 3 days and then washed once with culture medium, cultured without dexamethasone for 4 days, and then the treatment was repeated (□); with $4 \times 10^{-6}$ M dexamethasone for 3 days and then washed, cultured without dexamethasone for 7 days, and then the treatment was repeated (□); with $4 \times 10^{-5}$ M dexamethasone for 3 days and then washed and cultured without dexamethasone (▲); or without dexamethasone throughout the culture period (★). The culture medium was replaced by fresh medium at least once every 3 days. The cell density of the dexamethasone-treated cells was kept at $2 \times 10^{5}$-8 $\times 10^{5}$/ml.

The long-term effects on differentiation and proliferation of M1 cells of 3 different treatments with dexamethasone were also examined: at $1 \times 10^{-7}$ M continuously for 30 days, at $1 \times 10^{-6}$ M for 3 days/week, and at $4 \times 10^{-6}$ M for 3 days/10 days. Of these treatments, continuous treatment with $1 \times 10^{-7}$ M dexamethasone was the most effective for inhibiting cell proliferation, although the product of time and concentration was smallest with this treatment (Fig. 2). After 20 days, the cumulative cell number was reduced 10-fold by continuous treatment with $1 \times 10^{-7}$ M dexamethasone. The inhibitory effect of continuous treatment with dexamethasone was observed for more than 60 days (data not shown).

A proteinous inducer also induced differentiation of M1 cells into macrophage-like cells and decreased their proliferation (2). More than 90% of the cells became phagocytic when cultured with the proteinous inducer (10% conditioned medium of the peritoneum) for 3 days. The cell number of the inducer-treated culture increased until day 3, but then decreased. However, rapid reappearance of growing cells was observed even when the cells were treated with a high concentration of the inducer. The resistant cells were blastic and nonphagocytic. After about 2 weeks, the proliferation rate of the treated cells became similar to that of untreated cells (Fig. 3).

Effects of Combinations of 1α,25(OH)2D3 and Antileukemic Drugs on Proliferation of M1 Cells. We next examined the long-term effect of differentiation inducers and antileukemic drugs added simultaneously on growth and differentiation of M1 cells. As mentioned above, proliferation of M1 cells was markedly suppressed for 20 days by $24 \text{ nM} \ 1\alpha,25(\text{OH})_2\text{D}_3$ alone. However, after about 25 days growing cells reappeared and the proliferation rate of the treated cells became similar to that of untreated cells (Fig. 4). These resistant cells were blastic and nonphagocytic.

Table 2 Effect of continuous treatment with 1α,25(OH)2D3 and antileukemic drugs on the induction of differentiation of M1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>M1 cells phagocytic activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4 ± 2*</td>
</tr>
<tr>
<td>1α,25(OH)2D3</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>Daunomycin</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>ara-C</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>1α,25(OH)2D3 plus daunomycin</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>1α,25(OH)2D3 plus ara-C</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>Daunomycin plus ara-C</td>
<td>17 ± 4</td>
</tr>
</tbody>
</table>

* Mean ± SD.

The 1α,25(OH)2D3-resistant cells did not appear when M1 cells were cultured for 35 days continuously with $24 \text{ nM} \ 1\alpha,25(\text{OH})_2\text{D}_3$ plus 8.9 nm (5 ng/ml) daunomycin or with 24
Fig. 5. Growth arrest of 1α,25(OH)2D3-less sensitive M1 cells induced by long-term treatment with 1α,25(OH)2D3 and an antileukemic drug. 1α,25(OH)2D3-less sensitive cells (M1-LS) were cultured with 24 nM 1α,25(OH)2D3 (●), 8.9 nM daunomycin (▲), 36 nM ara-C (◇), 24 nM 1α,25(OH)2D3 plus 8.9 nM daunomycin (●) or 24 nM 1α,25(OH)2D3 plus 36 nM ara-C (◇), or without 1α,25(OH)2D3 and antileukemic drugs (○).

Fig. 6. Growth arrest of U-937 cells induced by long-term treatment with 1α,25(OH)2D3 and actinomycin D. U937 cells were cultured with 24 nM 1α,25(OH)2D3 (●), 0.87 nM actinomycin D (▲), or 24 nM 1α,25(OH)2D3 plus 0.87 nM actinomycin D (●), or without 1α,25(OH)2D3 and actinomycin D (○).

A more striking effect of 1α,25(OH)2D3 and an antileukemic drug in inhibiting cell proliferation was observed with a subclone of M1 cells that was less sensitive to 1α,25(OH)2D3 (Fig. 5). In this case, although 24 nM 1α,25(OH)2D3 alone did not induce clear growth arrest of M1 cells, treatment with 1α,25(OH)2D3 plus 36 nM ara-C or 1α,25(OH)2D3 plus 8.9 nM daunomycin induced complete growth arrest of the M1 cells after day 10 (Fig. 5). Similar effects of inducers and antileukemic drugs were observed when M1 cells were treated with 1α,25(OH)2D3 plus actinomycin D or dexamethasone plus actinomycin D (data not shown).

Effects of 1α,25(OH)2D3 and Actinomycin D on Proliferation of U937 Cells. Human monoblastic U937 cells are induced to differentiate into macrophages by 1α,25(OH)2D3 (18). The effects of long-term continuous treatments with 1α,25(OH)2D3 and actinomycin D added simultaneously on proliferation of U937 cells were examined. Continuous treatment with 24 nM 1α,25(OH)2D3 or 0.87 nM (1 ng/ml) actinomycin D caused significant growth inhibition but not complete growth arrest of U937 cells (Fig. 6). The treated cells were monocyctic but not mature macrophage-like cells (Fig. 7). On the other hand, combined treatment with 1α,25(OH)2D3 plus actinomycin D induced complete growth arrest of the cells: the cell number increased until day 5 but did not change thereafter (Fig. 6). The treated cells were viable and had a macrophage-like morphology (Fig. 7).

Effect of 1α,25(OH)2D3 and an Antileukemic Drug on Survival of Mice Inoculated with M1 Cells. The in vitro studies described above suggested that combined treatment with a differentiation inducer and an antileukemic drug should be more effective therapeutically than treatment with a differentiation inducer alone. Therefore, we examined whether simultaneous treatment with 1α(OH)D3 plus daunomycin could prolong the survival of syngeneic SL mice inoculated with M1 cells. We found previously that 1α(OH)D3 was more effective than 1α,25(OH)2D3 in increasing the survival of mice inoculated with M1 cells (10). Therefore, we used 1α(OH)D3 instead of 1α,25(OH)2D3. SL mice inoculated with 3 x 10^6 M1 cells were treated with 1α(OH)D3, daunomycin, or 1α(OH)D3 plus daunomycin 3 times a week. In treatment with 1α(OH)D3 or daunomycin alone, a dose of 50 pmol (20 ng; approximately 1 μg/kg) 1α(OH)D3 plus daunomycin 3 times a week (10) and a dose of 1.8 nmol (10 μg; approximately 500 μg/kg) daunomycin 3 times a week (Fig. 8) were optimum for prolonging survival of mice inoculated with M1 cells (Fig. 8). The mean survival time of untreated mice inoculated with M1 cells was about 19 days, while that of mice treated with 50 pmol 1α(OH)D3 or 18 nmol daunomycin 3 times a week was about 27 days; on the other hand, that of mice treated with 1α(OH)D3 plus daunomycin was about 35 days (Fig. 9). These results indicate that the combination treatment with 1α(OH)D3 and daunomycin is more effective therapeutically than treatment with either drug alone.

DISCUSSION

Continuous treatment of myeloid leukemia M1 cells with a low concentration of a differentiation inducer resulted in complete arrest of cell proliferation after several division cycles. This treatment is cytostatic rather than cytotoxic to leukemia cells. The concentration required to arrest cell proliferation completely was much less on continuous treatment than on intermittent treatment. Moreover, the concentration required in continuous treatment may be tolerable to many leukemic patients, although we should still be alert to the side effects of prolonged treatment with differentiation inducers in clinical use (3).
GROWTH CONTROL BY DIFFERENTIATION INDUCERS

Fig. 7. Effect of continuous treatment with 1α,25(OH)2D3 and actinomycin D on the induction of morphological differentiation of U937 cells. U937 cells were cultured for 20 days with 24 nM 1α,25(OH)2D3 (a), 0.87 nM actinomycin D (c), or 24 nM 1α,25(OH)2D3 plus 0.87 nM actinomycin D (d), or without 1α,25(OH)2D3 and actinomycin D (b). Cells were stained with May-Grünwald-Giemsa solution.

Fig. 8. Effects of various doses of daunomycin on the survival of mice inoculated with M1 cells. Mice were inoculated i.p. with 3 x 10⁶ M1 cells and given injections i.p. of various doses of daunomycin 3 times a week. Points and bars, mean ±SD for 6–10 mice.

Although proliferation of M1 cells in our long-term in vitro culture system was markedly suppressed by continuous treatment with a low concentration of a differentiation inducer, resistant cells appeared about 3 weeks after the start of treatment. On the other hand, no resistant cells appeared when M1 cells were treated continuously with the inducer (1α,25(OH)2D3 or dexamethasone) and a low concentration of an antileukemic drug (daunomycin, ara-C, or actinomycin D). A similar effect of inducer and antileukemic drug on proliferation in a human monoblast-like cell line U937 cells was also observed. Most of the U937 cells treated with the inducer and antileukemic drug differentiated into macrophages, and their growth was arrested for a long time. These results suggest that continuous treatment of myeloid leukemia cells with a combination of a differentiation inducer and a low dose of an antileukemic drug is effective for inducing terminal differentiation of cells and that the appearance of differentiation-resistant cells is suppressed or retarded by this combination treatment. These results also suggest the potential usefulness of combination therapy with a differentiation inducer and antileukemic drug for maintenance therapy after induction of complete remission in leukemia, since this treatment might induce terminal differentiation and growth arrest of the small number of leukemic cells remaining after remission induction therapy.

The mechanism of the combination effect of a differentiation inducer and an antileukemic drug is still unknown. Antileu-
kemic drugs such as actinomycin D, daunomycin, and ara-C are known to restore in vivo and in vitro sensitivity to differentiation induction of nondifferentiating M1 cells (19–22). Thus the antileukemic drugs used here may enhance the sensitivity of M1 cells and U937 cells to 1α,25(OH)2D3. In fact, the proportion of differentiated cells after treatment with 1α,25(OH)2D3 plus antileukemic drugs was significantly higher than that after treatment with 1α,25(OH)2D3 alone. Furthermore, if 1α,25(OH)2D3-resistant cells appear when M1 cells are treated with 1α,25(OH)2D3 and antileukemic drugs, they might be sensitized to 1α,25(OH)2D3 by the antileukemic drugs.

Daunomycin, ara-C, and actinomycin D have weak differentiation-inducing activity in M1 cells (6). Therefore, the effect of 1α,25(OH)2D3 and an antileukemic drug might be due to its combined effect with inducers which have different mechanisms of effects on induction of differentiation. In fact, the synergistic effect of a proteinous inducer and dexamethasone was observed in proteinous inducer-resistant M1 cells (data not shown). On the other hand, various compounds with different modes of action can induce differentiation of many types of myeloid leukemia cells, including freshly isolated leukemic cells from patients with acute myeloid leukemia (2). Thus, control of proliferation of leukemic populations that are resistant to a single compound may be achieved by exposure to a combination of several different inducers.

The present results clearly indicate the effectiveness of combination treatment with a differentiation inducer and an antileukemic drug on survival of mice inoculated with M1 cells. We used 1α(OH)D3 as an inducer instead of 1α,25(OH)2D3, since 1α(OH)D3 was more effective than 1α,25(OH)2D3 in increasing the survival of mice inoculated with M1 cells (10). Although 1α(OH)D3 was only one hundredth as active as 1α,25(OH)2D3 in suppressing cell growth and inducing differentiation of M1 cells in vitro, it was rapidly metabolized to the active form 1α,25(OH)2D3 when injected i.p. (10, 15). The maximal plasma level of 1α(OH)D3 was attained 3 h after its administration and was maintained for up to 12 h. The plasma level of 1α,25(OH)2D3 was more than 30% of the maximal level, even after 48 h (10). Therefore, we administered 1α(OH)D3 to mice 3 times a week. The effect of combination treatment with 1α(OH)D3 and daunomycin in increasing the survival time of mice inoculated with M1 cells was less than that of this combination treatment on induction of differentiation and suppression of cell proliferation in vitro. The reason for this difference is still unknown. Nevertheless, it should be stressed that the combination treatment with an inducer and an antileukemic drug could be more effective therapeutically than treatment with either an inducer or antileukemic drug alone.

On the other hand, there is a recent report that the combination of hexamethylene bisacetamide (a cell differentiation inducer) with cytotoxic agents such as ara-C and Adriamycin do not show synergistic cytotoxic effects in terms of human promyelocytic leukemia HL-60 cell growth, although the results are obtained from short-term (3 days) culture (23). The use of the long-term culture systems provides more detailed information about the time course of suppression of leukemic cell proliferation by various inducers, the frequency of appearance of inducer-resistant cells, and the combined effects of different inducers or an inducer plus an antileukemic drug on leukemic cell proliferation. This information should be useful in development of a suitable method for induction of terminal differentiation of leukemic cells for therapy of myeloid leukemia.

REFERENCES


Control of Proliferating Potential of Myeloid Leukemia Cells during Long-Term Treatment with Vitamin D₃ Analogues and Other Differentiation Inducers in Combination with Antileukemic Drugs: *In Vitro* and *in Vivo* Studies

Takashi Kasukabe, Yoshio Honma, Motoo Hozumi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/2/567

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.