Induction of Differentiation of WEHI-3B D⁺ Leukemic Cells Transfected With Differentiation-Stimulating Factor/Leukemia Inhibitory Factor Receptor cDNA

By Mikio Tomida

Differentiation-stimulating factor (D-factor)/leukemia inhibitory factor can induce the differentiation of mouse myeloid leukemia M1 cells and also stimulate proliferation of the interleukin-3 (IL-3)-dependent cell line, DA-1a. To determine whether D-factor can induce the differentiation of leukemia cells other than M1 cells, WEHI-3B D⁺ mouse myelomonocytic leukemia cells were transfected with a plasmid containing mouse D-factor receptor cDNA. Expression of D-factor receptor in transfected cells was determined by binding of [125I]D-factor and analyzed by Scatchard’s method. The transfected cells had high-affinity D-factor receptors with a dissociation constant of 100 to 200 pmol/L and binding sites per cell varied from 67 to 1,500 among several clones. The cells expressing a high level of D-factor receptor were induced to differentiate by D-factor; about 60% of the cells exhibited the ability to reduce nitroblue tetrazolium and expression of the differentiation antigen Mac-1 (CD11b) on the cell surface increased. The effect of cytokines, which induce differentiation of M1 cells, on the transfected WEHI-3B cells was examined. The sensitivity to oncostatin M was identical to that against D-factor in the cells of each clone. Expression of D-factor receptor in WEHI-3B cells promoted sensitivity to IL-6 and granulocyte colony-stimulating factor (G-CSF). Induction of differentiation of the cells accompanied the suppression of proliferation. Treatment of the cells with D-factor for longer than 5 days resulted in 50% inhibition of growth. These results indicate that the stimulating effect of D-factor on the differentiation of malignant myeloid cells is not unique to M1 cells.

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WE AND OTHERS¹⁻³ purified and cloned differentiation-stimulating factor (D-factor)/leukemia inhibitory factor as a cytokine inducing differentiation of mouse myeloid leukemia M1-T22 cells into macrophages. We found recently that injection of recombinant mouse D-factor prolonged the survival times of mice implanted with the M1-T22 cells⁴ and that human D-factor suppressed the proliferation of freshly isolated leukemic blast cells from acute myeloblastic leukemia patients.⁵ These results suggest that D-factor is of potential value in the therapy of myeloid leukemia. However, D-factor stimulates the proliferation of some leukemic cell lines, such as mouse DA-1a⁶ and human TF-1.⁷ Furthermore, it has a wide variety of biologic activities besides its actions on normal and leukemic hematopoietic cells.⁸

Gearing et al⁹⁺¹⁰ isolated a cDNA for human D-factor receptor and proved that D-factor receptor associates the IL-6 signal transducer, gp130. Oncostatin M (OSM) and D-factor share a common receptor complex and receptors for IL-11 and ciliary neurotrophic factor (CNTF) also share gp130.¹¹ The existence of a common signal transduction pathway, mediated through gp130, may explain the functional redundancy of D-factor, OSM, IL-6, IL-11, and CNTF.

To determine whether D-factor can induce the differentiation of leukemic cells other than M1-T22 cells, we cloned a cDNA encoding mouse D-factor receptor¹² and introduced the D-factor receptor into WEHI-3B D⁺ mouse myelomonocytic leukemia cells, which were induced to differentiate by IL-6 or granulocyte colony-stimulating factor (G-CSF), but not D-factor.¹³⁺¹⁴ We show here that D-factor and OSM induce differentiation and suppress proliferation of WEHI-3B D⁺ cells transfected with D-factor receptor cDNA.

MATERIALS AND METHODS

Cells and cell culture. WEHI-3B D⁺ leukemia cells (kindly provided by Dr Alan C. Sartorelli, Yale University School of Medicine, New Haven, CT) were cultured in McCoy’s 5A modified medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; GIBCO BRL or Flow Laboratories, Stanmore, Australia) in an atmosphere of 5% CO₂ in air at 37°C. Myeloid leukemic M1 cells were cultured as described previously.¹ Clone T-22 cells can be induced to differentiate by D-factor, but clone R-4 cells can not.

Cytokines. Recombinant mouse D-factor was produced in Chinese hamster ovary cells and recombinant human G-CSF was produced in Escherichia coli and purified to homogeneity as described previously.³⁻⁴ Recombinant human IL-6 was kindly provided by Ajinomoto Co (Kawasaki, Japan). Recombinant human OSM and IL-11 were purchased from R & D Systems, Minneapolis, MN.

Plasmid construction. Mouse cDNAs encoding the membrane receptor and the soluble receptor for D-factor were cloned from a cDNA library prepared from the liver of a pregnant mouse as described previously.¹³ The full length of cDNA encoding the membrane receptor was prepared by recombinant of the L51 clone and LS3 clone (in Fig 2A of reference 12) at the Thr111 restriction site. The cDNA was subcloned into the Eco R1 site of the expression vector, pSG5 (Stratagene, La Jolla, CA), which includes the SV40 early promotor. It was confirmed that the cDNA was functional, by transient expression of the cDNA in COS-7 cells. The binding of [125I]D-factor to COS-7 cells increased 80-fold after transfection of the cDNA into the cells.

Transfection of DNA. DNA was introduced into WEHI-3B cells by electroporation using the Gene Pulser (Bio-Rad Laboratories, Richmond, CA). The cells (1 × 10⁶) were suspended in 0.8 mL of HEPES-buffered saline (50 mmol/L HEPES, 137 mmol/L NaCl, 6.8 mmol/L KCl, 0.28 mmol/L Na₂HPO₄, and 0.1% dextrose, pH 7.1) containing 20 μg of linearized expression plasmid and 1 μg of pXT1 (Stratagene) conferring neomycin resistance, and incubated on ice for 15 minutes. The cells were exposed to a 450 V pulse with a capacitance of 960 μF, and returned to ice. After incubation on ice

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The cells were transferred into 96-well plates and cultured in medium 3 days. The cells were treated with D-factor the next day on which assays were performed as described previously. The specific radioactivity with blue-black formazan deposit was determined by examination of NBT and 100 ng/mL of 12-0-tetradecanoylphorbol 13-acetate at 37°C for 30 minutes. In the microscopic assay, the percentage of cells differentiated cells. However, no strict relationship existed between the degree of differentiation and the D-factor binding capacity of the cells. For example, although both clones had a similar D-factor binding capacity, clone DR6, but not clone DR3, responded to D-factor.

As another marker of differentiation, Mac-1 antigen on induce differentiation in mouse WEHI-3B leukemic colonies. Because the cells do not have D-factor receptors, D-factor receptor cDNA was introduced into the cells to determine the general effects of D-factor on leukemic cells. Mouse D-factor receptor cDNA was transfected into WEHI-3B cells, together with a plasmid carrying the neomycin resistance gene. G-418-resistant transformants were tested for the ability to bind 125I-D-factor, and several independent clones were isolated (Table 1). Neo cells were transfected with the neomycin resistance gene alone and did not express D-factor binding sites. The specific binding of D-factor to two clones of M1 cells is also shown in Table 1. M1-T22 cells differentiate in response to D-factor, whereas M1-R4 cells do not respond to the cytokine. The properties of the D-factor binding to these stable transformants were examined. Clones of DR2 and DR8 were incubated with 125I-D-factor at various concentrations. Figure 1 shows the binding characteristics of D-factor to DR2 cells. Scatchard analysis showed that the cells have 1,500 binding sites per cell with a dissociation constant of 111 pmoL. The number of D-factor receptors expressed in clone DR8 was 67 sites per cell with a dissociation constant of 20/ pmoL (data not shown).

Effects of cytokines on the proliferation and differentiation of the transfected cells. The relationship between the ability of cells to reduce NBT and the specific 125I-D-factor binding capacity of the transfected WEHI-3B cells was examined. The cells were incubated with or without D-factor one day after the assay of D-factor-binding of the cells. After 3 days of incubation, the number of cells and the ability of the cells to reduce NBT, a differentiation marker, were determined. Similar results were obtained from three independent experiments, and a typical result from one experiment was summarized in Table 1. The increased expression of the receptor resulted in enhancement of the ability of the cells to differentiate. However, no strict relationship existed between the degree of differentiation and the D-factor binding capacity of the cells. For example, although both clones had a similar D-factor binding capacity, clone DR6, but not clone DR3, responded to D-factor.

As another marker of differentiation, Mac-1 antigen on

**Table 1. D-Factor Binding and D-Factor-Dependent Differentiation of WEHI-3B Cells Transfected With D-Factor Receptor cDNA**

<table>
<thead>
<tr>
<th>Clone</th>
<th>D-Factor Binding (cpm)</th>
<th>D-Factor -</th>
<th>D-Factor +</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR1</td>
<td>12,016 ± 145*</td>
<td>6.7 ± 2.7</td>
<td>62.8 ± 7.0</td>
</tr>
<tr>
<td>DR2</td>
<td>15,851 ± 411</td>
<td>5.6 ± 2.4</td>
<td>58.2 ± 8.2</td>
</tr>
<tr>
<td>DR3</td>
<td>1,565 ± 83</td>
<td>0.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>DR4</td>
<td>4,165 ± 74</td>
<td>7.5 ± 1.5</td>
<td>62.3 ± 5.0</td>
</tr>
<tr>
<td>DR5</td>
<td>923 ± 1</td>
<td>2.6 ± 0.5</td>
<td>21.8 ± 2.3</td>
</tr>
<tr>
<td>DR6</td>
<td>2,008 ± 59</td>
<td>8.5 ± 1.5</td>
<td>55.4 ± 5.3</td>
</tr>
<tr>
<td>DR7</td>
<td>1,574 ± 12</td>
<td>6.0 ± 2.0</td>
<td>35.0 ± 10</td>
</tr>
<tr>
<td>DR8</td>
<td>630 ± 37</td>
<td>0.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>Neo</td>
<td>−14 ± 15</td>
<td>0</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>M1-T22</td>
<td>4,014 ± 216</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M1-R4</td>
<td>2,047 ± 90</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Cells (10⁶ in 0.5 mL) were incubated with [125I]D-factor (5 ng) for 3 hours at room temperature. Specific binding was determined by subtracting the binding in the presence of 60-fold excess of unlabelled D-factor. NBT-reducing cells were determined by the microscopic assay after treatment of the cells with or without 10 μg/mL of D-factor for 3 days. The cells were treated with D-factor the next day on which D-factor binding was determined.

Abbreviation: ND, not determined.

* Each value is the mean of duplicate determinations ± SE.

for 5 minutes, the cells were cultured in 20 mL medium for 2 days. The cells were transferred into 96-well plates and cultured in medium containing G-418 at the final concentration of 2 mg/mL.

**Cellular binding of 125I-D-factor.** Iodination of mouse D-factor with chloramine-T and assay of cellular binding of 125I-D-factor were performed as described previously. The specific radioactivity of the 125I-D-factor preparation was 3.4 × 10⁶ counts per minute (cpm)/μg protein.

**Properties of differentiated cells.** The ability of cells to reduce nitroblue tetrazolium (NBT) was used as a functional marker of differentiation. Cell numbers were determined using a Model ZM Coulter Counter (Coulter Electronics, Luton, UK). One × 10⁶ cells were incubated in 1 mL of serum-free medium containing 0.1% NBT and 100 ng/mL of 12-O-tetradecanoylphorbol 13-acetate at 37°C for 30 minutes. In the microscopic assay, the percentage of cells with blue-black formazan deposit was determined by examination of a minimum of 200 cells. In the colorimetric assay, the reaction was stopped by adding HCl to the final concentration of 1 mol/L. The formazan suspension was centrifuged and the formazan deposits were solubilized by adding dimethyl sulfoxide. The absorption of the formazan solution at 560 nm was measured with a spectrophotometer (U-2000, Hitachi, Tokyo).

Expression of the granulocyte- and macrophage-specific antigen Mac-1 (CD11b) on the cell surface was determined by indirect immunofluorescent staining and flow cytometry as described previously. The cells (2 × 10⁶) were washed with phosphate-buffered saline (PBS) and incubated in 50 μL of rat anti-Mac-1 monoclonal antibody (diluted 1:20; Boehringer Mannheim Biochemica, Indianapolis, IN) in PBS containing 0.1% bovine serum albumin (BSA) on ice for 30 minutes. Then the cells were washed and stained in 50 μL of phycoerythrin-conjugated goat-antirat IgG antibody (diluted 1:20; Cedar Lane, Ontario, Canada) in PBS containing 0.1% of BSA on ice for 30 minutes. The cells were washed, then analyzed in an Epics Elite flow cytometer (Coulter Electronics).

**RESULTS**

Establishment of cell lines expressing the D-factor receptor. Both G-CSF and IL-6, but not D-factor, were able to

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**Fig 1. Binding of 125I-D-factor to DR2 cells. (A) Specific binding of 125I-D-factor to cells incubated at increasing concentrations of the radiolabeled ligand. (B) Scatchard plot of the binding data.**
EXPRESSION OF D-FACTOR RECEPTOR ON WEHI-3B

Fig. 2. Expression of Mac-1 antigen on DR1 cells treated with D-factor. (A) Cells were treated with 10 ng/mL of D-factor for 6 days, then stained with monoclonal anti-Mac-1 and phycoerythrin-conjugated goat-antirat IgG antibody. (B) Untreated cells. (C) Untreated cells stained with the antirat IgG alone.

The induction of differentiation of the transfected cells accompanied a decrease in cell number. The cell number of clones DR1, DR2, DR4, and DR6 treated with D-factor for 3 days was 70% to 80% of that of the control culture. The growth rates of clones DR3, DR8, and Neo were not changed by treatment of the cells with D-factor. Figure 3 shows the effect of D-factor on proliferation of DR2 cells. Treatment of the cells with D-factor for longer than 5 days resulted in 50% inhibition of growth. A similar inhibitory effect of D-factor on the proliferation of DR1 cells was observed (data not shown).

Next, the effects of several cytokines, which induced differentiation of mouse myeloid leukemia M1 cells, on the WEHI-3B cells transfected with D-factor receptor cDNA were examined. Results are shown in Table 2. DR1, DR2, and DR6 are D-factor-sensitive clones, whereas DR8, Neo, and parental WEHI-3B are not. All clones responded to IL-6. IL-6 is the most effective inducer against parental WEHI-3B and the cells transfected with neomycin resistance gene alone (Neo). Parental WEHI-3B cells responded poorly if at all to the inducing activity of G-CSF in liquid culture as reported by Li et al.18 Some clones, which responded to D-factor, were more sensitive to G-CSF, as compared with the parental cells. The sensitivity to IL-11 was varied among these clones. The sensitivity to OSM was identical to that against D-factor in the cells of each clone. The growth inhibition was also observed in clones that were induced to differentiate by OSM. Effects of various concentrations of D-factor or OSM on clones DR1 and DR2 were examined. Figure 4 shows both D-factor and OSM induced the differentiation of DR1 cells in a similar dose-dependent manner. Both cytokines were effective at concentrations higher than 0.5 ng/mL. The stimulating effects of both cytokines were saturated at concentrations lower than 5 ng/mL. IL-6, IL-11, and G-CSF were more effective at the concentration of 100 ng/mL than at 10 ng/mL (data not shown).

DISCUSSION

WEHI-3B cells transfected with D-factor receptor cDNA were induced to differentiate by treatment of the cells with D-factor or OSM. Recently, Gearing et al.19 reported that cotransfection of D-factor receptor cDNA and gp130 cDNA into the IL-3-dependent pro-B cell line, BAF-B03, resulted in D-factor- and OSM-induced proliferation. These results confirmed that D-factor can both induce differentiation and stimulate proliferation, depending on the cell line. BAF-B03 cells, but not WEHI-3B cells, required cotransfection of gp130 cDNA. This discrepancy comes from the difference in the expression of endogenous gp130 in both cell lines. Parental WEHI-3B cells respond to IL-6 and the cells transfected with D-factor receptor cDNA alone express high affinity D-factor receptors, indicating the expression of endogenous gp130 in WEHI-3B cells.

All three transfected WEHI-3B clones, which expressed a higher number of D-factor receptors than those of M1-T22 cells, were sensitive to D-factor (Table 1). On the other hand, all transfected clones examined, which showed lower capacity of D-factor-binding than that of DR8 clone, could not be induced to differentiate by D-factor (data not shown). Therefore, the level of D-factor receptor expression in these leukemia cells seemed to be important for a response to D-
factor. However, a borderline of D-factor binding capacity in determination of D-factor responsiveness was not clear-cut. Variability in D-factor responsiveness in the transfected clones was observed. The result suggests that the number of the receptor alone does not determine the ability of differentiation of the cells. Alternatively, it is possible that the degree of expression of D-factor receptor varied during the experiments, although the assay of D-factor binding and the differentiation of the cells were performed within several days. The expression of the receptor on the transfected cells continued for more than 1 month after the isolation of the clones, but the degree of the expression fluctuated during long-term culture. Li et al\textsuperscript{18} also reported that the increased expression of G-CSF receptor in WEHI-3B cells transfected with the receptor cDNA resulted in the induction of differentiation of the cells in liquid culture.

We previously showed that D-factor is a potent inducer of differentiation of M1-T22 cells and that G-CSF is a weak inducer for the cells.\textsuperscript{15} Combination of G-CSF with D-factor resulted in synergistic stimulation of differentiation.\textsuperscript{29} IL-6 and OSM also induced differentiation of M1-T22 cells.\textsuperscript{29} Although IL-11 and IL-6 showed the same effect in many cases, IL-11 did not induce the differentiation of the cells (unpublished results, December 1992). Rose and Bruce\textsuperscript{23} compared the structure of the proteins and genes of D-factor, OSM, IL-6, and G-CSF and suggested that these four cytokines have evolved from a common ancestor and are members of a single cytokine family. Furthermore, receptors for D-factor, OSM, IL-6, and IL-11 share a signal transduction subunit, gp130.\textsuperscript{11} On the other hand, IL-6 and G-CSF, but not D-factor, induce the differentiation of parental WEHI-3B cells.\textsuperscript{13,14} Transfected WEHI-3B cells with D-factor receptor cDNA respond to D-factor, OSM, IL-6, and G-CSF (Table 2). Some transfected clones, but not all clones, responded to IL-11. Expression of D-factor receptor in WEHI-3B cells promoted the sensitivity to IL-6 and G-CSF and a small portion of these cells had the ability to reduce NBT in the

![Fig 4. Induction of differentiation of DR1 cells by D-factor or OSM. Cells were exposed to various concentrations of D-factor (C) or OSM (D) for 4 days and the percentages of NBT-reducing cells were determined. Points and bars are means ± SE for duplicate determinations.](image-url)
absence of treatment of any cytokines (Table 1). These reasons are unknown at present. It is possible that that FCS contains a low level of D-factor or OSM and that these cytokines stimulate differentiation of the WEHI-3B cells synergistically with G-CSF or IL-6. It was shown that induction of differentiation of M1-T22 cells by IL-6 or G-CSF increased in the presence of a low concentration of D-factor.13,20

Although D-factor at 0.5 ng/mL induced differentiation and suppressed proliferation of M1-T22 cells, other leukemic cell lines, such as HL-60 and U-937, respond poorly to D-factor.22 We recently showed that D-factor suppressed the proliferation of freshly isolated leukemic blast cells from acute myeloblastic leukemia patients.3 The present study showed that D-factor inhibited the proliferation of WEHI-3B cells if they expressed an adequate number of D-factor receptors. These results indicate that D-factor suppresses proliferation of various myeloid leukemia cells.

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REFERENCES

Induction of differentiation of WEHI-3B D+ leukemic cells transfected with differentiation-stimulating factor/leukemia inhibitory factor receptor cDNA

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