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Effect of Recombinant Human D-Factor on the Growth of Leukemic Blast Progenitors from Acute Myeloblastic Leukemia Patients

Guan-Jin Chen,[1] Mikio Tomida,[2] Motoo Hozumi[2] and Nobuo Nara[1, 3]

[1]*Laboratory Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113 and* [2]*Department of Chemotherapy, Saitama Cancer Center Research Institute, Ina, Kitaadachi-gun, Saitama 362*

We studied the effects of D-factor on the growth of leukemic blast progenitors from 15 patients with acute myeloblastic leukemia and two leukemia cell lines in methylcellulose and suspension cultures. When stimulated by granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor or interleukin-3, leukemic blast progenitors undergo terminal division with limited differentiation in methylcellulose culture, forming blast colonies. Leukemic blast progenitors can renew themselves. The self-renewal can be detected as secondary colony formation after replating primary blast colonies in fresh methylcellulose media and by the growth of clonogenic cells in suspension culture. D-Factor suppressed primary and secondary colony formation in methylcellulose culture. Furthermore, D-factor suppressed clonogenic cell recovery in suspension culture. The suppression by D-factor of the growth of leukemic blast progenitors was not significantly dependent upon the colony-stimulating factors used as growth-stimulating factors. High concentration of G-CSF did not overcome the suppressive effect of D-factor. The results indicate that D-factor is effective in suppressing not only terminal division but also self-renewal of leukemic blast progenitors.

Key words: D-factor — Acute myeloblastic leukemia — Leukemic blast progenitor — Self-renewal — Leukemia cell line

Murine D-factor has been purified as a factor inducing differentiation into macrophages and granulocytes of murine myeloid leukemic M1 cell line.[1)] D-Factor shows various effects on myeloid leukemia cell lines, and has recently been proved to be identical with leukemia inhibitory factor (LIF) named by Hilton *et al.*[2)] Human gene encoding D-factor/LIF has been cloned using a synthetic oligonucleotide probe designed on the basis of the murine D-factor sequence.[3)] Recombinant human D-factor has been revealed to inhibit the ^{3}H-thymidine incorporation of murine M1 cell line and also to induce phagocytosis. If recombinant human D-factor is active for the inhibition of human leukemic cells, D-factor could provide a new approach to leukemia therapy.

Acute myeloblastic leukemia (AML) is a clonal hemopathy that probably originates at some stage of the hematopoietic differentiation pathway. The progressive accumulation of leukemic cells is supported by a minor subpopulation of leukemic blast progenitors. Leukemic blast progenitors have the characteristics of stem cells; they may renew themselves and/or undergo terminal division with limited differentiation.[4, 5)] The growth of leukemic blast progenitors, which is stimulated by granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3), can be assessed in terms of blast colony-formation in methylcellulose culture and by the clonogenic cell recovery in suspension culture. In the present study, we examined the effect of recombinant human D-factor on the self-renewal and terminal division of leukemic blast progenitors from 15 AML patients. Furthermore, the effect of D-factor on the G-CSF-dependent human acute myeloid cell line OCI/AML1a and the IL-3-dependent human lymphoid cell line TMD2 was studied. The results demonstrate that D-factor is effective in inhibiting not only terminal division but also self-renewal of leukemic blast progenitors.

MATERIALS AND METHODS

Cells Peripheral blood was taken from 15 AML patients after obtaining informed consent. The diagnosis was based on the morphology, cytochemistry, phenotype and cytogenetics according to the FAB classification.[6)] The characteristics of the patients are shown in Table I. Mononuclear cells were separated through density gradient centrifugation (1.077 g/ml). T-Lymphocytes were then depleted using sheep erythrocytes. After this procedure, more than 90% of the obtained cells were myeloblasts. Cells were either used immediately or kept frozen until use at -80°C in α-minimal essential medium (α-MEM, Gibco, Grand Island, NY) with 10% dimethylsulfoxide and 50% fetal calf serum (FCS, Gibco).

Two established leukemia cell lines were used in the present study. One is OCI/AML1a cell line established from an AML M4 patient.[7)] This cell line depends exclu-

[3] To whom correspondence should be addressed.

Table I. Patients' Characteristics

Patient No.	Age (yr)	Sex	Diagnosis (FAB)[a]	Peripheral blood		Bone marrow	Treatment[c]	Outcome[d]
				WBC (/μl)	Blasts (%)	Blasts (%)		
1	37	M	M3	1,300	6	90	MAD	CR
2	55	F	M4	16,400	30	78	BHAC-DMP	F
3	51	F	M4	66,300	95	91	BHAC-DMP	F
4	68	M	M1	21,700	66	90	BHAC-DMP	CR
5	57	M	M4	5,500	6	47	MAD	CR
6	54	F	M3	3,200	80	93	BHAC-DMP	CR
7	44	F	M2	3,200	17	40	BHAC-DMP	CR
8	63	M	M4	1,800	61	61	MAD	CR
9	45	F	M4	10,900	30	78	BHAC-DMP	F
10	47	F	M1	150,000	96	70	BHAC-DMP	CR
11	61	M	M1	198,800	98	67	MAD	CR
12	67	M	M3	2,100	8	90	MAD	CR
13	64	M	M4	61,000	4	52	MAD	F
14	44	M	M4	7,800	33	90	DCMP	CR
15	54	F	M1	21,900	86	NA[b]	MAD	under Tx

a) FAB: French-American-British classification.[6]
b) NA=not assessed because of dry marrow tap.
c) MAD=6-mercaptopurine, cytosine arabinoside, and daunorubicin. BHAC-DMP=behenoyl cytosine arabinoside, daunorubicin, 6-mercaptopurine, and prednisolone. DCMP=daunorubicin, cytosine arabinoside, 6-mercaptopurine, and prednisolone.
d) CR=complete remission, F=failure, Tx=treatment.

sively on G-CSF for its optimal growth. The other is TMD2 cell line, established from a patient with chronic lymphocytic leukemia in the acute phase.[8] Although TMD2 cell line is not a myeloid leukemia cell line, this cell line has a unique character of IL-3-dependent growth. Therefore, TMD2 cell line was included in the present study to test the effect of D-factor on leukemic cells.

Biological reagents Recombinant human D-factor was obtained as medium conditioned by CHO cells transfected with a plasmid containing cDNA encoding human D-factor.[3] Purified recombinant human D-factor (0.5 ng/ml) induced 50% phagocytic cells in M1-T22 cell cultures. Recombinant human G-CSF and GM-CSF were kindly supplied by Chugai Pharmaceutical Co., Tokyo, and Hoechst Japan, Tokyo, respectively. Recombinant human IL-3 was purchased from Genzyme, Cambridge, MA.

Methylcellulose culture T Cell-depleted mononuclear cells obtained from AML patients or two cell lines were cultured in methylcellulose media to assess clonogenicity. Then 10^4 cells were plated in 0.1 ml of α-MEM with 20% FCS and 0.8% methylcellulose. As a leukemic blast growth stimulating factor, G-CSF, GM-CSF, or IL-3 was added at the concentration of 10 ng/ml. D-Factor was added at several concentrations to the culture. The culture was incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After incubation for 7 days, colonies containing more than 20 cells were counted under an inverted microscope. The plating efficiency was shown as PE1.

In some instances, 4×10^5 cells were plated in 1 ml of methylcellulose medium with 10 ng/ml G-CSF and various concentrations of D-factor. After incubation for 7 days, colonies were counted, pooled, and washed three times in α-MEM with 10% FCS. The cells obtained (10^4 cells) were plated in 0.1 ml of α-MEM with 20% FCS, 0.8% methylcellulose and 10 ng/ml G-CSF. The culture was incubated for 7 more days. Colonies of more than 20 cells were scored under an inverted microscope. The secondary colony plating efficiency was designated as PE2.

Suspension culture Cells were also cultured in suspension by the method described previously with a minor modification.[9] Briefly, 3×10^6 cells were cultured in 3 ml of α-MEM with 20% FCS and 10 ng/ml G-CSF. D-Factor was added at several concentrations. After incubation for 7 days at 37°C in a humidified atmosphere of 5% CO_2 in air, cells were collected, counted, washed three times, and then plated in methylcellulose media to determine clonogenic cells. The recovery of clonogenic cells in suspension was calculated by multiplying the number of cells recovered in suspension by the number of colonies determined in methylcellulose assay.

Statistics Data are shown as the mean±SD of three to five replicate cultures. Group data were compared by using Student's *t* test.

RESULTS

D-Factor was added at several concentrations to methylcellulose culture of leukemic blast progenitors. Figure 1 shows typical results in 3 patients. Although the effect of D-factor on colony formation by leukemic blast progenitors varied among the patients, D-factor significantly suppressed blast colony formation stimulated by G-CSF, GM-CSF, or IL-3 in a dose-dependent manner. Since primary blast colony formation reflects the terminal division of leukemic blast progenitors, the results indicate that D-factor is effective in suppressing the terminal division of leukemic blast progenitors. It is also of interest to determine the effect of D-factor on the self-renewal capacity of leukemic blast progenitors, because the self-renewal capacity is characteristic of leukemic blast progenitors.[4] For this purpose, we further studied the effect of D-factor on the secondary colony formation in methylcellulose by leukemic blast progenitors and also on the recovery of clonogenic cells in suspension culture, since these assay systems are suitable to examine the self-renewal of leukemic blast progenitors.[9]

Figure 2 shows the effect of D-factor on primary (PE1) and secondary (PE2) colony formation by leukemic blast progenitors and on clonogenic cell recovery in suspension culture, where G-CSF was used as a growth stimulating factor. D-Factor suppressed PE1, PE2 and clonogenic cell recovery in suspension culture in a dose-dependent manner. D-Factor at 100 ng/ml suppressed PE1 to 30–1.5% of the control, PE2 to 40–0.8%, and clonogenic cells recovered per dish in suspension culture to 80–10%. It also suppressed the growth of OCI/AML1a cell line and TMD2 cell line (Fig. 3). Primary colony-forming cells are considered to be the cell population mainly undergoing terminal division. Secondary colony-forming cells and clonogenic cells growing in suspension culture are considered to be a self-renewing cell population. The results shown in Figs. 2 and 3, therefore, suggest that D-factor is effective for the suppression of not only cells undergoing terminal division but also cells capable of self-renewal.

However, a carry-over effect of D-factor must be ruled out to confirm the effect of D-factor on the self-renewal capacity of leukemic blast progenitors. In PE2 assay, leu-

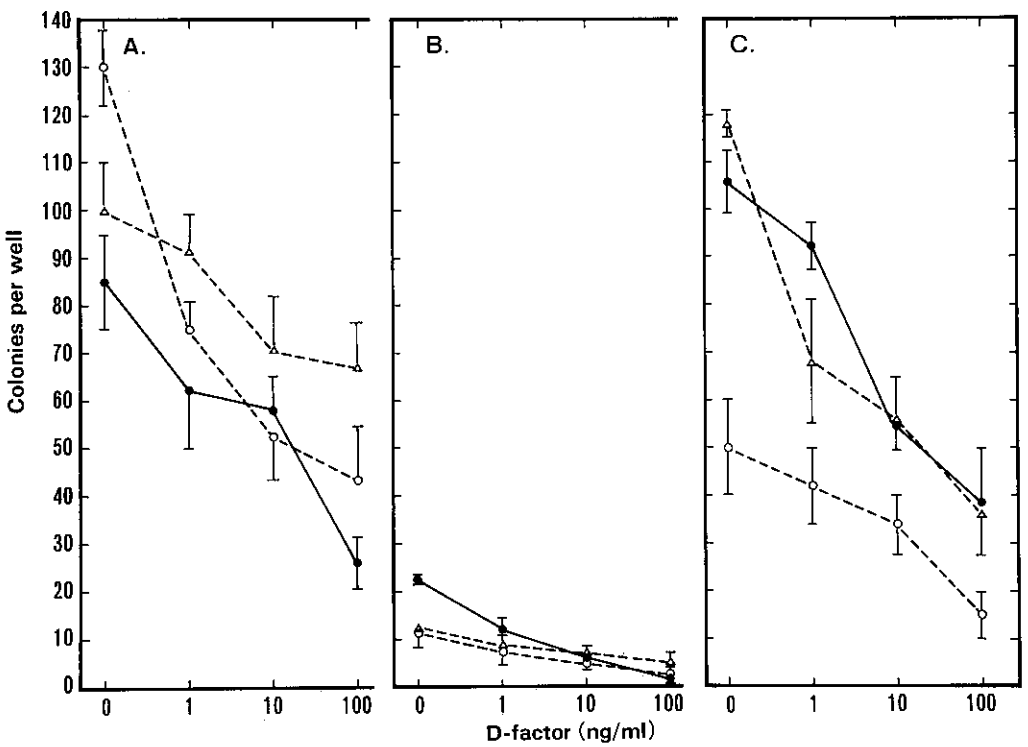

Fig. 1. Effects of D-factor on blast colony formation in methylcellulose culture stimulated by 10 ng/ml of G-CSF (●), GM-CSF (○), or IL-3 (△). The stimulating effects of the CSFs varied among the patients. D-Factor suppressed the blast colony formation in a dose-dependent manner regardless of the type of supplemented CSF. Typical results in three patients (A, B, C) are shown. Data are given as the mean±SD of five replicate cultures.

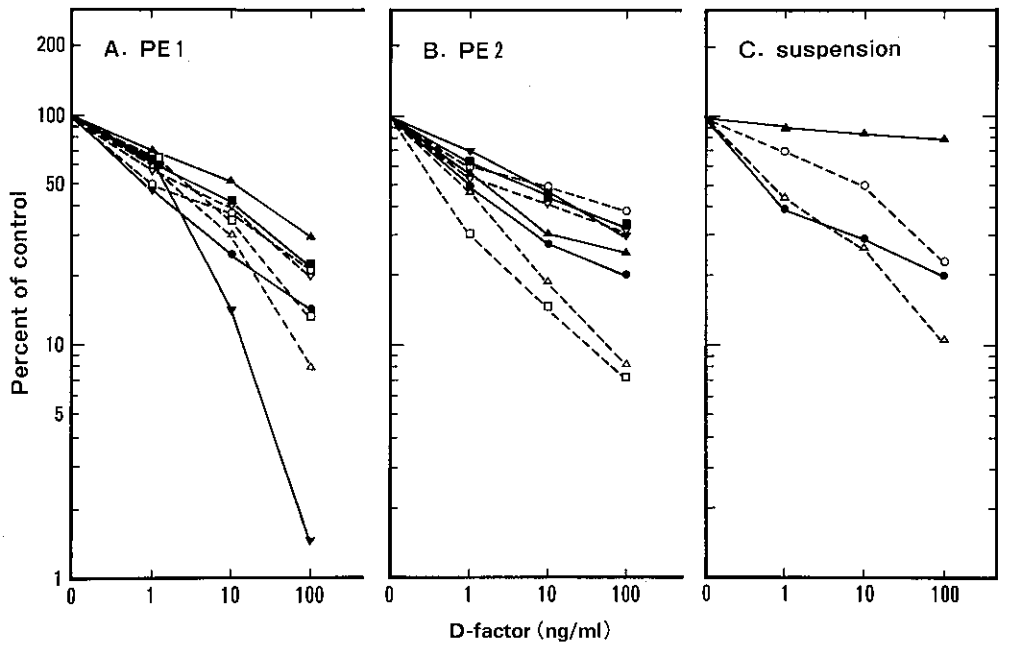

Fig. 2. Effects of D-factor on primary (PE1; A) and secondary (PE2; B) colony formation by leukemic blast progenitors in methylcellulose culture and the recovery of clonogenic cells in suspension culture (C). The same symbol shows results from the same patient. The data are given as percent of control.

kemic cells were exposed to D-factor in methylcellulose for 7 days, and then were replated in fresh methylcellulose medium without D-factor. If D-factor were carried over in leukemic cells, the residual D-factor would affect the secondary colony formation. The same situation was considered in suspension culture. To rule out such a carry-over effect, the following experiments were done.

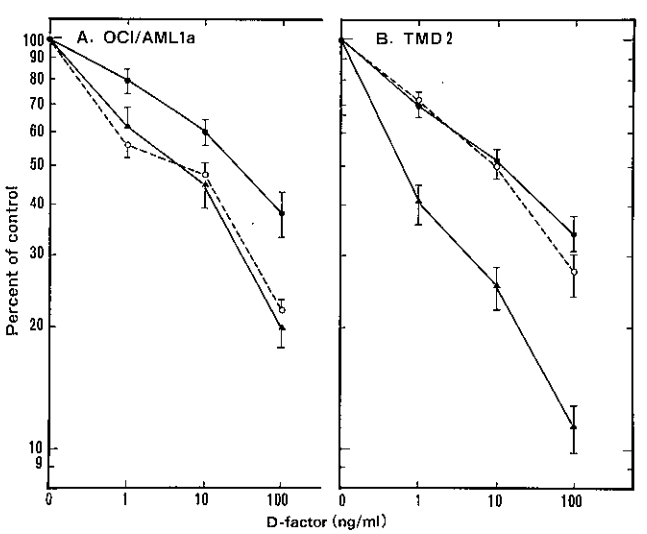

Fig. 3. Effects of D-factor on the growth of the G-CSF-dependent leukemia cell line OCI/AML1a (A) and the IL-3-dependent leukemia cell line TMD2 (B). D-Factor suppressed PE1 (●), PE2 (○), and clonogenic cell growth in suspension culture (▲) of both cell lines stimulated by G-CSF and IL-3, respectively.

Leukemic cells were exposed to 10 ng/ml of D-factor in suspension culture for 7 days, washed three times, and irradiated at 30 Gy to destroy their proliferative capacity. Then irradiated cells were added to either methylcellulose or suspension culture, where intact leukemic cells were plated at various cell concentrations to provide fixed numbers of cells in culture. As a control, intact leukemic cells were cultured with or without the addition of irradiated leukemic cells not pre-exposed to D-factor. Irradiated cells did not grow themselves, but supported the growth of intact cells possibly through cell-to-cell interaction.[10] The effect of irradiated cells did not differ between the cells pre-exposed to D-factor and the cells not pre-exposed (data not shown). The results demonstrate that D-factor was not carried over in the leukemic cells.

To confirm further the effect of D-factor on the self-renewal capacity of leukemic blast progenitors, we added

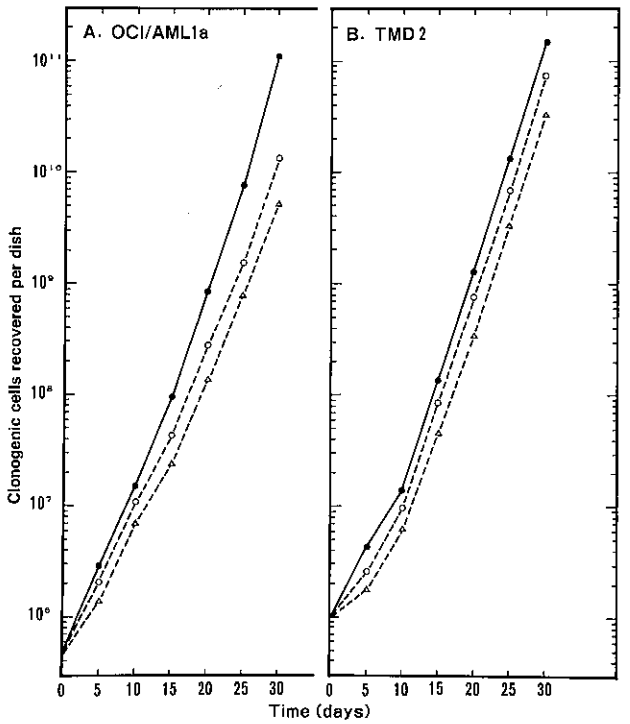

Fig. 4. Long-term culture of OCI/AML1a (A) and TMD2 (B) cell lines. Leukemic cells were cultured in suspension for 5 days and used for clonogenic assay in methylcellulose to determine clonogenic cells. Cells were repeatedly subcultured (every 5th day), and cumulative growth curves of clonogenic cells were drawn. Control (●); D-factor 1 ng/ml (○); D-factor 10 ng/ml (△).

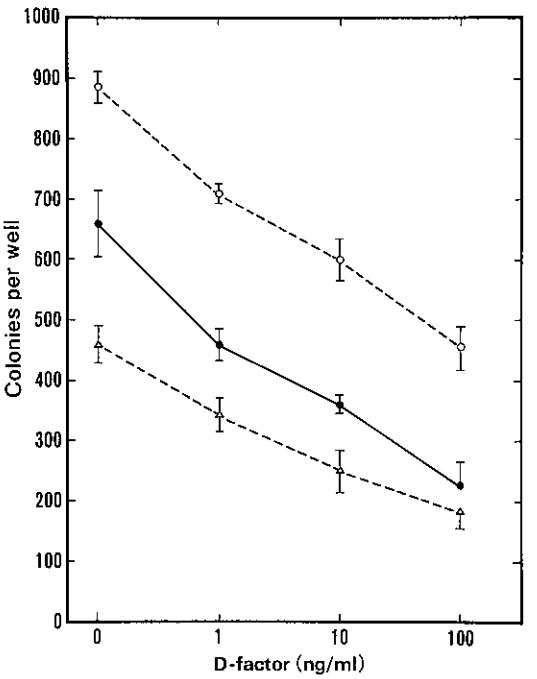

Fig. 5. Suppressive effect of D-factor on blast colony formation stimulated by 1 ng/ml (△), 10 ng/ml (●), or 100 ng/ml (○) of G-CSF.

D-factor repeatedly to a long-term culture of leukemic cells. Figures 4A and 4B show the results in OCI/AML1a cell line and TMD2 cell line, respectively. OCI/AML1a and TMD2 cell lines grew exponentially for years in the presence of G-CSF and IL-3, respectively, reflecting their high self-renewal capacity. When 1 and 10 ng/ml of D-factor were continuously added to long-term cultures of the cell lines, the growth of the cell lines was suppressed. The suppression of the growth of OCI/AML1a cell line was more prominent upon longer exposure to D-factor. This result supports the conclusion that D-factor was effective in the suppression of the self-renewal capacity of OCI/AML1a cell line.

Finally, we tested whether the suppression of the growth of leukemic blast progenitors by D-factor could be overcome by the addition of excess amounts of growth-stimulating factor. Figure 5 shows the effects of D-factor on the growth of leukemic blast progenitors stimulated by increasing concentrations of G-CSF. G-CSF has been shown to stimulate the growth of leukemic blast progenitors in most AML patients.[11] D-Factor showed a similar suppressive effect on leukemic blast progenitors stimulated by 1–100 ng/ml of G-CSF. Thus, excess G-CSF did not overcome the suppressive effect of D-factor on leukemic blast progenitors, indicating that the growth inhibition of leukemic blast progenitors by D-factor cannot be explained in terms of a lack of growth-stimulating factor.

DISCUSSION

D-Factor suppressed the blast colony formation by leukemic blast progenitors from AML patients and two leukemia cell lines (Figs. 1–3). D-Factor was reported to induce the differentiation to macrophages and granulocytes of murine leukemia M1 cell line.[1] Therefore it is possible that D-factor induces the differentiation of leukemic blast progenitors and reduces blast colony formation. To examine this possibility, we investigated the change in morphology of leukemic cells cultured in suspension. D-Factor did not seem to induce significant morphologic change in leukemic cells stained with Wright's stain during culture for as long as 7 days (data not shown). Although longer-term culture would be necessary to confirm the differentiation-inducing effect of D-factor in leukemic cells, the suppressive effect of D-factor on leukemic blast progenitors may be explained by

growth inhibition of leukemic blast progenitors rather than by the induction of differentiation into mature cells. D-Factor suppressed not only PE1 but also PE2 and clonogenic cells recovered in suspension culture (Figs. 2 and 3). Because PE2 and the recovery of clonogenic cells in suspension culture have been considered to reflect the self-renewal of leukemic blast progenitors,[9] the results support the conclusion that D-factor also affects the cell population capable of self-renewal.

Self-renewal capacity has been described as a characteristic of leukemic blast progenitors and may be an important factor contributing to the clinical outcome of AML patients.[4] Therefore, AML therapy must be directed to inhibit the self-renewal of leukemic blast progenitors in order to eradicate leukemic blast progenitors and cure AML patients. On the basis of the present data, D-factor may be useful as an agent to suppress the self-renewal of leukemic blast progenitors in AML patients. D-Factor by itself, however, may not be effective to cure AML patients considering that leukemic cells grew even in the continuous presence of 10 ng/ml of D-factor (Fig. 4). To apply D-factor for clinical use in the treatment of AML, suitable combination therapy with other antileukemic drugs should be sought.

The growth of leukemic blast progenitors is regulated by a network of several cytokines.[12] An excess of G-CSF did not overcome the suppressive effect of D-factor on leukemic blast progenitors (Fig. 5). This result suggests that D-factor directly affects leukemic cells. However, it is possible that D-factor induces the production and secretion of some other growth-negative cytokines, such as tumor necrosis factor-α and transforming growth factor-β, by leukemic cells. For example, interferon-γ has been reported to enhance the expression of the tumor necrosis factor-α gene in leukemia cells.[13] To determine the precise mechanism by which D-factor inhibits the leukemic cell growth, the interaction between cytokines affecting the growth of leukemic blast progenitors should be clarified.

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