Induction of Differentiation of Human Leukemia Cells by Various Combinations of Cytokines and Low-Molecular-Weight Inducers[1)]

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To explore agents for differentiation therapy of leukemias, various combinations of cytokines and low-molecular-weight inducers were examined for differentiation-inducing activity toward three kinds of human leukemia-derived cell lines. The strongest differentiation inducing activity on promyelocytic HL60 cells and histiocytic U937 cells was obtained by combining recombinant tumor necrosis factor (rTNF), interferon-γ (IFN-γ), retinoic acid (RA), and 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$D$_3$). For myeloblastic ML1 cells, the combination of rTNF, IFN-γ, and RA had the strongest differentiation-inducing activity.

Keywords differentiation; leukemia cell; cytokine; synergistic effect

Attention has increasingly been focused on differentiation therapy of myeloid leukemia using differentiation-inducing compounds. For this purpose, the ideal would be compounds that induce 100% of the leukemia cell population to differentiate, since, if any leukemia cells survive, they will proliferate and ultimately kill the host. We found that mouse serum containing tumor necrosis factor (TNF),[2)] which was obtained from mice injected first with Bacillus Calmette-Guerin and 2 weeks later with lipopolysaccharide (LPS), has strong differentiation-inducing activity toward mouse myeloid leukemia M1 cells.[3)] Since the effect of human recombinant TNF (rTNF) on the differentiation of M1 cells was much weaker than that of the serum containing TNF, we suggested that other cytokines in the serum acted synergistically with TNF in inducing differentiation.[3)] The differentiation-inducing ability of TNF toward human myeloid leukemia HL60 cells was reported by Takeda *et al.*[4)] and Trinchieri *et al.*,[5)] who showed that TNF-induced differentiation of HL-60 cells was synergistically enhanced by interferon-γ (IFN-γ).[5)] The combination of TNF and IFN-γ also synergistically induced differentiation of human leukemia ML-1 cells.[6)] Synergism between TNF and 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$D$_3$) in inducing differentiation of HL60 cells has been reported by Trinchieri *et al.*[7)] Tamatani *et al.*[8)] reported that TNF in the conditioned medium of pig peritoneal macrophages stimulated with LPS was able to induce differentiation of M1 cells only in the presence of interleukin 1α (IL-1α). Recently, combinations of 1α,25(OH)$_2$D$_3$ or 1α-hydroxyvitamin D_3 and antileukemia drugs such as 1-β-D-arabinofuranosylcytosine (Ara-C), actinomycin D (Act-D), and daunomycin have been used for differentiation therapy of M1 cells in mouse.[9)] Combination treatment of myelomonocytic leukemia (c-WRT-7)-injected rats with LPS and daunomycin completely inhibited the development of leukemia cells in all of the treated rats.[10)]

In the present study, we examined combinations of various differentiation-inducing compounds with the aim of obtaining the most effective possible differentiation of human leukemia cells. Our strategy was: first, to find the combination(s) of cytokines with the strongest differentiation-inducing activity; second, to find the most effective combination(s) of low-molecular-weight compounds to induce marked differentiation; and finally, to combine the most effective cytokines with the most effective low-molecular-weight inducers to obtain the strongest possible differentiation-inducing activity.

Materials and Methods

Materials Retinoic acid (RA), Ara-C,[2)] Act-D, and dexamethasone (DEX) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Prostaglandin E_2 (PGE_2) was from Ono Pharmaceutical Co. (Osaka). 1α,-25(OH)$_2$D$_3$ was kindly donated by Chugai Pharmaceutical (Tokyo), Human recombinant IL-α (rIL-1α, 10^8 U/mg) was obtained from Genzyme Corporation (Boston, U.S.A.). Human recombinant TNF (rTNF, 1.1×10^8 U/mg) was kindly provided by Fujisawa Pharmaceutical Co. (Osaka). Human recombinant IFN-β (rIFN-β, 10^8 U/mg) was a gift from Toray Ind. Inc. (Tokyo). Human IFN-α, IFN-β and IFN-γ were purchased from Japan Chemical Research Co., Ltd. (Tokyo). Eagle's medium was purchased from Gibco Laboratories (New York, U.S.A.).

Cells and Cell Culture Human leukemia HL60, ML1, and U937 cells were kindly supplied by Dr. K. Takeda of Showa University and grown in RPMI 1640 medium (Flow Laboratories, Inc.) supplemented with 10% (v/v) fetal calf serum at 37 °C under 5% CO_2 in air.

Assay for Differentiation-Inducing Activity Leukemia cells (1×10^5 cells/ml) were treated with the sample solution containing various concentrations of differentiation inducing compounds for 3 d. The differentiation inducing activity was monitored by measuring the ability of the cells to reduce nitroblue tetrazolium (NBT) as described previously.[3,11)] After incubation of the cells with NBT for 20 min at 37 °C, they were examined microscopically. Phagocytic activity was determined by measuring the capacity of the cells to engulf polystyrene latex particles (average diameter, 0.81 μm; Difco Laboratories). Leukemia cells (10^6 cells/ml) were suspended in serum-free Eagle's minimum essential medium containing 0.2% latex particles and incubated at 37 °C for 4 h. After incubation, the cells were washed once with phosphate-buffered saline. Cells containing more than 10 latex particles were scored as phagocytic cells. Smear preparations were cytochemically stained for α-naphthyl acetate esterase and AS-D chloroacetate esterase as described before.[11)]

Results

Effects of Combinations of Cytokines and Inducers on HL60 Cells As shown in Fig. 1, rTNF (1.7×10^4 U/ml) alone induced differentiation of human promyelocytic HL60 cells. In accordance with the results reported by Trinchieri *et al.*[7)] the combination of rTNF (1.7×10^4 U/ml) plus IFN-γ (100 U/ml) synergistically induced differentiation of HL-60 cells. Neither rIl-1α (0.28 U/ml), IFN-α (100 U/ml), rIFN-β (100 U/ml) nor rG-CSF (100 ng/ml) significantly affected HL60 cell differentiation. Therefore, the combination of rTNF and IFN-γ was used as a differentiation inducing cytokine for HL60 cells.

Figure 2 shows the effects of the combination of rTNF (1.7×10^3 U/ml) plus IFN-γ (100 U/ml) with various low-molecular-weight inducers on the differentiation of HL60

cells. The left side of each column shows the differentiation-inducing activity of the low-molecular-weight inducers and their combinations. Although $1\alpha,25(OH)_2D_3$ (10^{-9} M) alone induced marked differentiation of HL60 cells, the concentration of $1\alpha,25(OH)_2D_3$ was lowered to 10^{-10} M to show more clearly the effect of combinations. The right side of each column shows the effect of the combination of rTNF plus IFN-γ plus the low-molecular-weight inducers.

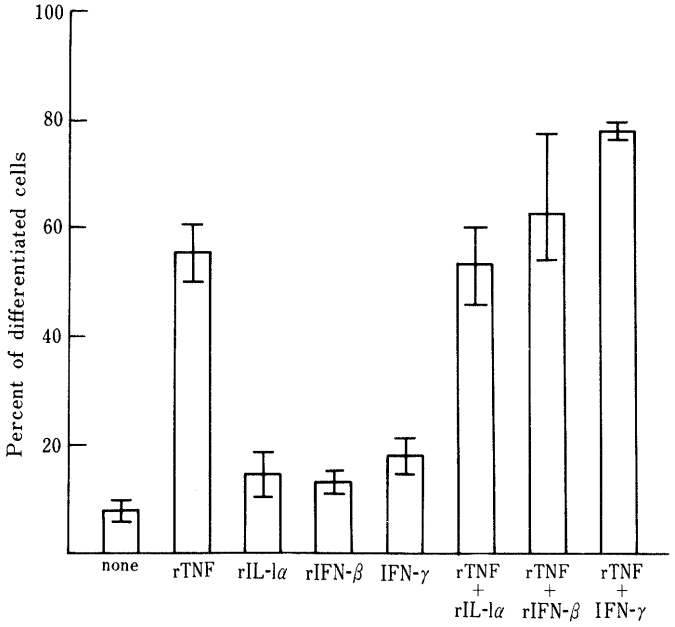

Fig. 1. Differentiation of HL60 Cells Induced by Combinations of Cytokines

HL60 cells were cultured in the presence of rTNF (1.7×10^4 U/ml), rIL-1α (0.28 U/ml), rIFN-β (5.71×10^3 U/ml), or IFN-γ (100 U/ml) alone or in the presence of various combinations of these cytokines for 2 d. Differentiation-inducing activity of these cytokines or their combinations was measured by NBT staining as described in the text. Each value is the mean ± S.D. of triplicate determinations.

The combination of five inducers, rTNF plus IFN-γ plus RA plus DEX plus $1\alpha,25(OH)_2D_3$, was most effective in inducing differentiation in HL60 cells and about 90% of the cells were induced to differentiate, while a combination of four inducers, rTNF plus IFN-γ plus 1α, $25(OH)_2D_3$ plus

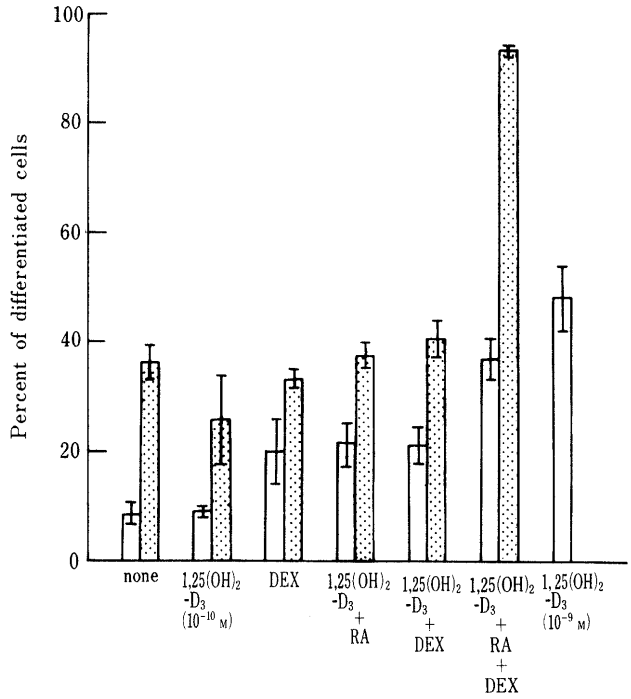

Fig. 2. Differentiation of HL60 Cells Induced by Combinations of Low-Molecular-Weight Inducers in the Absence (Open Bars) or in the Presence (Hatched Bars) of rTNF + IFN-γ

Differentiation-inducing activity of these cytokines or their combinations were measured by NBT staining as described in the text. Each value is the mean ± S.D. of triplicate determinations. Concentrations of cytokines and low-molecular-weight inducers were: rTNF, 1.7×10^3 U/ml; IFN-γ, 100 U/ml; $1\alpha,25(OH)_2D_3$, 10^{-10} M; DEX, 10^{-6} M; RA, 10^{-5} M.

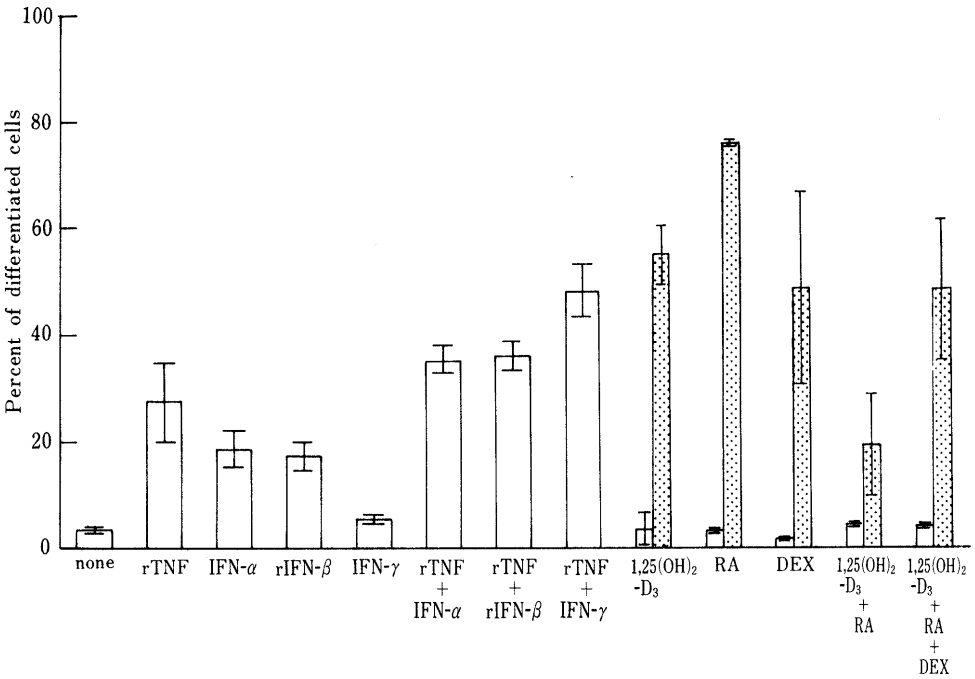

Fig. 3. Differentiation of ML1 Cells Induced by Combinations of Low-Molecular-Weight Inducers in the Absence (Open Bars) or in the Presence (Hatched Bars) of rTNF + IFN-γ

Differentiation-inducing activity of these cytokines or their combinations was measured by NBT staining as described in the text. Each value is the mean ± S.D. of triplicate determinations. Concentrations of cytokines and low-molecular-weight inducers were: rTNF, 1.2×10^4 U/ml; IFN-γ, 100 U/ml; rIFN-β, 5.7×10^3 U/ml; IFN-γ, 100 U/ml; $1\alpha,25(OH)_2D_3$, 10^{-9} M; RA, 10^{-5} M; DEX, 10^{-6} M.

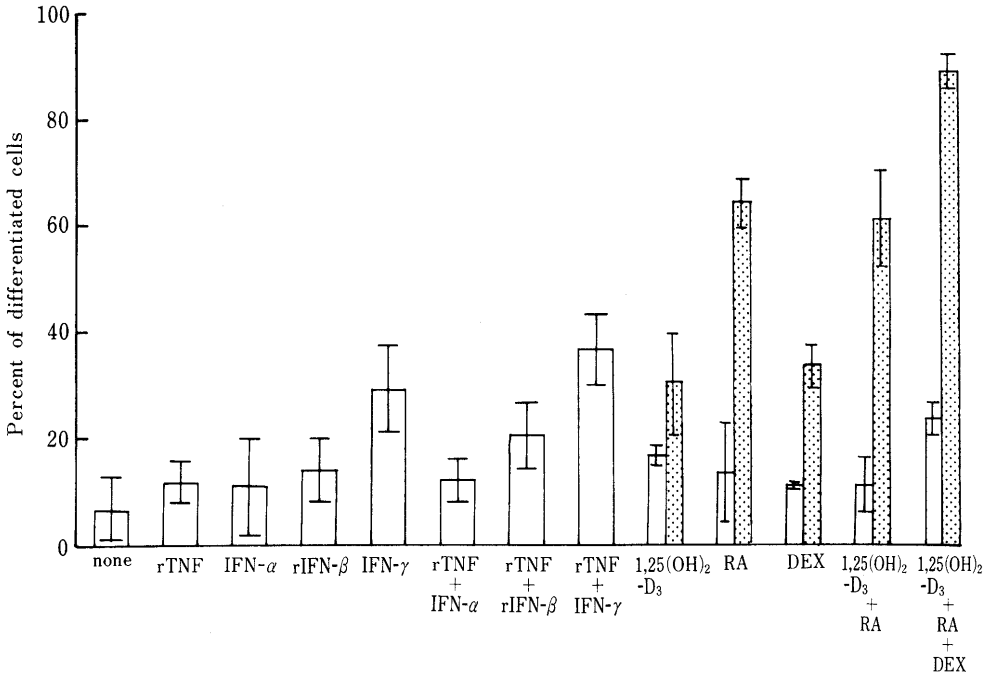

Fig. 4. Differentiation of U937 Cells Induced by Combinations of Low-Molecular-Weight Inducers in the Absence (Open Bars) or in the Presence (Hatched Bars) of rTNF+IFN-γ

Differentiation-inducing activity of these cytokines or their combinations was measured by NBT staining as described in the text. Each value is the mean±S.D. of triplicate determinations. Concentrations of cytokines and low-molecular-weight inducers were: rTNF, 1.7×10^3 U/ml; IFN-α, rIFN-β, and IFN-γ, 70 U/ml; $1\alpha,25(OH)_2D_3$, 10^{-9} M; RA, 10^{-5} M; DEX, 10^{-6} M.

RA, induced 40% differentiation. Although DEX alone is reported to be a poor inducer of differentiation of HL60 cells,[12] its combination with rTNF plus IFN-γ plus $1\alpha,25(OH)_2D_3$ induced striking differentiation. This suggests that DEX can stimulate the differentiation of HL60 cells, although the mechanism is still unknown. Antileukemic drugs such as Act-D or Ara-C were not so effective in inducing differentiation of HL60 cells when combined with rTNF and IFN-γ (results not shown).

Effects of Combinations of Cytokines and Inducers on U937 and ML1 Cells When human myeloblastic ML1 cells was treated with either rTNF (1.2×10^4 U/ml), IFN-α (100 U/ml), rIFN-β (5.7×10^3 U/ml), or IFN-γ (100 U/ml), rTNF had the strongest differentiation inducing activity and IFN-γ had the smallest (Fig. 3). Among various combinations of cytokines, rTNF plus IFN-γ had the most synergistic effect. Therefore, the combination of rTNF and IFN-γ was used in combination with the low-molecular-weight inducers. As is evident from Fig. 3, low-molecular-weight inducers such as $1\alpha,25(OH)_2D_3$, RA and DEX alone or their combinations could not induce differentiation of the ML1 cell line used in the present study. However, the combination of rTNF, rIFN-γ, and RA (10^{-5} M) had the strongest differentiation-inducing activity. $1\alpha,25(OH)_2D_3$ in combination with rTNF and rIFN-γ had only an additive effect. It was unexpected that addition of $1\alpha,25(OH)_2D_3$ to the combination of rTNF, IFN-γ, and RA would lower the differentiation-inducing activity below that of rTNF, IFN-γ, and RA. The combination of five inducers, rTNF, IFN-γ, $1\alpha,25(OH)_2D_3$, RA, and DEX was less effective than the combination of rTNF, IFN-γ, and RA.

As shown in Fig. 4, human histiocytic U937 cells were only marginally induced to differentiate by either rTNF (1.7×10^3 U/ml), IFN-α (70 U/ml), or rIFN-β (70 U/ml) but were significantly induced by IFN-γ (70 U/ml). The combination of rTNF and IFN-γ showed additive differentiation-inducing activity for U937 cells. Among low-molecular-weight inducers either alone or in combination, $1\alpha,25(OH)_2D_3$ (10^{-9} M) plus RA (10^{-5} M) plus DEX (10^{-6} M) induced differentiation in about 24% of U937 cells. When rTNF and IFN-γ were combined with either RA or $1\alpha,25(OH)_2D_3$ plus RA, they induced marked differentiation of U937 cells. The strongest differentiation-inducing activity was observed on combined treatment of the cells with rTNF, IFN-γ, $1\alpha,25(OH)_2D_3$, RA, and DEX and 90% of the treated cells showed NBT-reducing ability.

TABLE I. Characteristics of HL60, ML1 and U937 Cells Treated with Combinations of Inducers

	HL60	ML1	U937
Growth inhibition[a]	98.0± 1.0	48.7±9.6	54.6±1.2
NBT reducing ability[b]	72.6± 6.8	76.0±0.7	82.6±5.5
	(11.9± 1.0)	(3.7±0.6)	(11.4±5.8)
Phagocytic ability[c]	46.0± 8.1	26.1±3.9	76.3±2.8
	(14.3± 4.8)	(11.8±1.2)	(8.9±2.1)
α-Naphthol acetate esterase[d]	47.0±10.3	50.8±9.1	90.8±1.4
	(4.5± 2.9)	(5.2±0.7)	(5.7±7.1)
Naphthol AS-D chloro-acetate esterase[e]	64.5± 0.5	35.1±3.5	55.7±5.7
	(5.6± 0.2)	(8.1±3.5)	(21.4±6.7)

HL-60 cells were treated with the combination of rTNF (1.7×10^3 U/ml), IFN-γ (100 U/ml), RA (10^{-5} M), $1\alpha,25(OH)_2D_3$ (10^{-10} M), and DEX (10^{-6} M) for 2 d. ML1 cells were treated with rTNF (1.2×10^4 U/ml)+IFN-γ (100 U/ml)+RA (10^{-5} M) for 2 d. U937 cells were treated with rTNF (1.7×10^3 U/ml)+IFN-γ (70 U/ml)+RA (10^{-5} M)+$1\alpha,25(OH)_2D_3$ (10^{-9} M)+DEX (10^{-6} M) for 2 d. Values are means±S.D. of 3 determinations. *a*) Percent of control. Cell number was counted and compared with the control cells without treatment. *b*) Percent of cell with NBT reducing ability. *c*) Percent of cells with phagocytic ability. *d*) Percent of positive cells with α-naphthyl acetate esterase activity. *e*) Percent of positive cells with naphthol AS-D chloroacetate esterase activity.

Characteristics of Differentiation Induced in Leukemia Cells by Combined Treatment with Cytokines and Inducers Table I shows the characteristics of the differentiation

induced in HL60, ML1, and U937 cells when these cells were treated for 2 d with the combinations of cytokines and low-molecular-weight inducers that showed the strongest differentiation-inducing activity. HL60 and U937 cells were treated with rTNF plus IFN-γ plus 1α,25(OH)$_2$D$_3$ plus RA plus DEX. Growth of HL60 cells thus treated was almost completely inhibited. Phagocytosis of $46.0 \pm 8.1\%$ of the treated cells was observed. Of the HL60 cells treated under these conditions, $47.0 \pm 10.3\%$ reacted with α-naphthyl acetate esterase activity stain and $64.5 \pm 0.5\%$ with the naphthol AS-D chloroacetate esterase activity stain, indicating that the treated cells were induced to differentiate into both granulocytic and monocytic cells. This result is understandable because TNF and 1α,25(OH)$_2$D$_3$ are known to induce differentiation towards monocytes[7,13-16] and RA towards granulocyte.[17-19]

In the case of treated U937 cells, growth inhibition was almost half ($54.6 \pm 1.2\%$) and phagocytic activity was $76.3 \pm 1.2\%$. Almost all treated U937 cells ($90.8 \pm 1.4\%$) were stained with α-naphthyl acetate esterase activity stain. It is not clear why naphthol AS-D chloroacetate esterase staining was increased from $21.4 \pm 6.7\%$ to $55.7 \pm 5.7\%$ by the treatment with the combined inducers.

ML1 cells were treated with rTNF plus IFN-γ plus RA. The treated ML1 cells showed NBT reducing ability of $76.0 \pm 0.7\%$ and growth of the treated cells was inhibited by $48.7 \pm 9.6\%$. Phagocytic activity of the treated cells was not so high ($26.1 \pm 3.9\%$). The cells were stained by both α-naphthol acetate esterase and naphthol AS-D chloroacetate esterase activity stain.

Discussion

In the case of HL60 cells, rTNF[4,5] and IFN-γ[20,21] by themselves and in combination[5] are known to induce differentiation. RA induces differentiation of HL60 cells into granulocyte-like cells,[17-19] while 1α,25(OH)$_2$D$_3$ induced the cells into monocyte (macrophage)-like cells.[13-16] By contrast, DEX is known to have no effect on the differentiation of HL60 cells.[12] It was, therefore, unexpected that the combination of DEX plus 1α, 25(OH)$_2$D$_3$ plus rTNF plus IFN-γ was more potent in inducing differentiation of HL60 cells than the combination of 1α,-25(OH)$_2$D$_3$, rTNF and IFN-γ. One possible explanation for this is that DEX-receptors are involved in the differentiation of HL60 cells by multiple inducers. The most potent differentiation-inducing activity for HL60 cells was obtained with a combination of rTNF, IFN-γ, 1α,25(OH)$_2$D$_3$, RA and DEX.

The combination of rTNF, IFN-γ, 1α,25(OH)$_2$D$_3$, RA and DEX was also the most potent for inducing differentiation of histiocytic U937 cells. U937 cells are induced to differentiate by IFN-α, IFN-β, RA, and 12-*O*-tetradecenoylphorbol 13-acetate towards monocyte-like cells.[22,23] Trinchieri *et al.*[7] demonstrated that the combination of TNF and 1α,25(OH)$_2$D$_3$ or IFN-γ, and that of IFN-γ and 1α,25(OH)$_2$D$_3$ synergistically induced differentiation of U937 cells.

The ML1 cell line used in the present study was insensitive to low-molecular-weight inducers including RA. However, in accordance with the finding of Takuma *et al.*[6] the combination of TNF and IFN-γ shows a synergistic effect on the differentiation of ML1 cells. The most effective differentiation-inducing activity for ML1 cells was observed with the combination of rTNF, IFN-γ, and RA. We can not explain at the present stage of our investigation why the differentiation-inducing activity for ML1 cells of the combination of rTNF, IFN-γ, RA, 1α,25(OH)$_2$D$_3$, and DEX is less than that of rTNF, IFN-γ, and RA.

Antileukemic drugs such as Act-D, Ara-C and daunomycin are often used in combination with differentiation-inducing drugs in leukemic therapy. When HL60, ML1, or U937 cells were treated with the combination of rTNF plus IFN-γ plus antileukemic drugs such as Ara-C and Act-D, the differentiation-inducing activity was less than that of rTNF, IFN-γ, DEX, and 1α,25(OH)$_2$D$_3$ combined or rTNF, IFN-γ, 1α,25(OH)$_2$D$_3$ and RA combined.

The present results demonstrate that 3 drugs are necessary to obtain the strongest differentiation-inducing effect on the leukemia cells examined. Although we examined only three kinds of human leukemia cell lines (promyelocytic HL60, myeloblastic ML1, and histiocytic U937 cells), rTNF, IFN-γ, and RA showed strong differentiation-inducing activity for all these leukemia cells. If more useful combinations of cytokines and inducers with stronger differentiation-inducing activity and fewer side effects can be found, they may be very useful in combination therapy of leukemia.

References and Notes

1) This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.
2) Abbreviations: Act-D, actinomycin D; Ara-C, 1-β-D-arabinofuranosylcytosine; 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; DEX, dexamethasone; rG-CSF, recombinant granulocyte-colony-stimulating factor; rIL-1α, recombinant interleukin 1α; IFN, interferon; rIFN-β, recombinant interferon-β; LPS, lipopolysaccharide; NBT, nitroblue tetrazolium; PGE$_2$, prostaglandin E$_2$; RA, retinoic acid; rTNF, recombinant tumor necrosis factor.
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