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EFFECT OF FIBROBLAST-DERIVED DIFFERENTIATION INDUCING FACTOR ON THE DIFFERENTIATION OF HUMAN MONOCYTOID AND MYELOID LEUKEMIA CELL LINES

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SUMMARY: A fibroblast-derived differentiation inducing factor (F-DIF) purified from medium conditioned by a human fibroblast cell line (WI-26VA4) induced differentiation of human monocytic leukemia cell lines (U-937, THP-1) into cells with macrophage characteristics. F-DIF alone induced the differentiation of ML-1 cells only marginally, but it synergistically increased the differentiation when combined with TNF. Interferon-γ, tumor necrosis factor, GM-CSF, interleukin-1 and interlukin-4 synergistically enhanced the differentiation of U-937 cells when combined with F-DIF.

Human myelogenous leukemic cells can be induced to differentiate into the monocyte/macrophage pathway by protein inducers called differentiation inducing factors (DIFs) in media conditioned by mitogen-stimulated human peripheral blood leukocytes (1-6). Some of these DIFs were recently purified and identified as interferon-γ (IFN-γ) and tumor necrosis factor (TNF) (7,8). Granulocyte colony stimulating factor (G-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF) have been shown to induce macrophage differentiation of promyelocytic HL-60 cells (9,10).

Abbreviations: F-DIF, fibroblast-derived differentiation inducing factor; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; CSF, colony stimulating factor; G-CSF, granulocyte-CSF; GM-CSF, granulocyte/macrophage-CSF; NBT, nitroblue tetrazolium.

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A fibroblast-derived differentiation inducing factor (F-DIF) that we have recently purified from medium conditioned by a human fibroblast cell line, WI-26VA4, might be interleukin-6 (Noda, H. et al. unpublished work). The N-terminal amino acid sequence was consistent with that of B-cell stimulatory factor 2 (IL-6) except for the absence of an N-terminal proline.

In the present study we examined effects of the purified F-DIF on the induction of differentiation of monocytoid (U-937 and THP-1) and myeloid (ML-1 and HL-60) leukemia cell lines. Combination effects of F-DIF and other cytokines such as interferons, interleukins and colony stimulating factors were also examined.

MATERIALS AND METHODS

Highly purified natural and recombinant IFNs were donated by The Green Cross Co., Osaka, Shionogi Pharmaceutical Co., Osaka and Toray Co., Tokyo, Japan. Recombinant human TNF (rHuTNF) was donated by Dainippon Pharmaceutical Co., Osaka, Japan. Recombinant human interleukin-1 (rHuIL-1) was donated by Otsuka Pharmaceutical Co., Tokushima, Japan. rHuIL-2 was donated by Shionogi Pharmaceutical Co., Osaka, Japan. rHuIL-3 and rHuIL-4 were purchased from Genzyme Co., Boston, Mass. rHuGM-CSF was a gift from Sumitomo Pharmaceutical Co. Osaka, Japan. rHuGM-CSF was a gift from Kirin Brewery Co., Tokyo, Japan.

Preparation of purified F-DIF: F-DIF was purified to homogeneity from conditioned medium of WI-26VA4, a human fibroblast line, by using chromatography. The purified protein was homogeneous by the criteria of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and N-terminal sequence analysis.

Cell lines: We used 4 cell lines in this study; U-937 (a monoblastic leukemia)(11), THP-1 (A monoblastic leukemia cell line)(12), ML-1 (a myeloblastic leukemia cell line)(13), and HL-60 (a promyelocytic leukemia cell line)(14).

Cell culture: Leukemic cell lines were maintained as suspension cultures in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum. Incubation was carried out at 37°C in a humidified 5% CO_2 incubator. Cells were cultured in a 96-well microplate by adding 0.2 ml of RPMI 1640 medium containing 6×10^4 cells, 10% FBS and test materials at the desired concentrations. The effects of the F-DIF and cytokines on cell growth and cell viability were assayed on a portion of the cell suspension on day three. Cell number was determined with a hematocytometer, and viability was estimated by trypan blue dye exclusion.

Differentiation assays: Differentiation was monitored by determining the appearance and accrual of various cellular markers normally associated with maturation of the monocytic elements.

NBT reducing ability: NBT-reducing activity was assayed by the modified method of Baehner and Nathan (15); $6x10^4$ cells were suspended in 0.2 ml of RPMI 1640 medium containing 10% FBS, 0.1% NBT dye and 30 ng TPA in a 96 well microplate for 60 minutes at 37°C in a 5% CO_2 incubator.
Colorimetric assay: After CO_2 incubation, the reaction was stopped by adding 50 µl 2N HCl to each well of the 96 well microplate and cooling on ice for 30 minutes. the medium was discarded, the formazan deposits were dissolved by adding 100 µl dimethylsulfoxide, and the dissolved formazan was measured in a spectrophotometer for 96 well microplates at optical density 590.
Microscopic assay: The percentage of cells containing blue-black formazan deposits was determined by counting at least 200 cells.

Phagocytic activity: Phagocytic activity was determined by the method of baker's yeast digestion (16). Cells were washed and mixed with a 0.2 ml opsonized yeast particle suspension and incubated for 1 hour at 37°C. Cells were washed again and 0.2 ml 0.01% fuchsin solution was added to the cell pellet. Viable cells that digested yeast particles were scored as phagocytosis positive, and at least 200 viable cells were counted in each determination.

Fc receptor activity: assay of the appearance of Fc receptors was performed by standard techniques for erythrocyte-antibody rosette formation, using sheep erythrocytes coated with rabbit antisheep erythrocyte antibodies (17).

Morphological differentiation: The appearance of morphological differentiation was assessed in stained slide preparations. Slides were prepared by centrifugation in a Cytospin. The slides were fixed in May-Gruenwald's solution for 3 minutes and stained with Giemsa solution for 30 minutes. Differential counts were made with a light microscope.

Esterase activity: Cytochemical staining for α-naphthyl acetate esterase and chloroacetate esterase activity was performed as previously described (18).

RESULTS

Dose-dependent effect of F-DIF: F-DIF, purified from conditioned medium of a human fibroblast cell line WI-26VA4, dose dependently induced NBT-reducing activity of U-937 cells. F-DIF was less inhibitory against the proliferation of U-937 cells (data not shown).

Effects of combination of F-DIF and TNF on the appearance of differentiation associated characteristics in U-937 cells: As shown in Table I, 10 ng/ml F-DIF induced Fc receptor activity and phagocytic activity. F-DIF treated cells were identified morphologically as intermediate stages of macrophage-like cells that had increased cytoplasm, aboundant granules, and an eccentrically placed oblate nucleus with loosely stranded nuclear

Table I

Effects of combination of F-DIF and TNF on the appearance of differentiation-associated characteristics of U-937

Addition	Fc receptor (%)	NBT reduction (%)	Phago-cytosis (%)	Non-specific esterase (%)
None	18	5	9	3
F-DIF 10 ng/ml	34	10	45	79
TNF 1 ng/ml	44	35	45	19
F-DIF 10 ng/ml +TNF 1 ng/ml	67	80	78	90

Fc receptor activity was measured on day one. NBT-reducing activity and phagocytic activity were determined on day three. Non specific esterase(α-naphthyl acetate esterase) activity was assayed on day six. Values are mean of three determinations.

chromatin. The activity of macrophage specific α-naphthyl acetate esterase was increased, and the activity of granulocyte specific chloroacetate esterase was decreased. A combination of 1 ng/ml TNF and 10 ng/ml F-DIF induced various differentiation associated markers, synergistically.

Effects of F-DIF and TNF, alone or incombination, on induction of NBT-reducing activity in various human myelogenous leukemic cell lines: As shown in Fig. 1, F-DIF induced NBT reducing activity of U-937 and THP-1 cells. On the other hand it only marginally induced differentiation of ML-1 and HL-60 cells. However, when combined with TNF, F-DIF synergistically induced NBT-reducing activity of ML-1 cells.

Combination effects of F-DIF and other cytokines: IFN-γ, either alone or incombination with F-DIF, remarkably induced the NBT-reducing activity of U-937 cells, while INF-α, and -β were inactive either alone or in combination with F-DIF. IL-1, -2, -3, or -4 alone induced NBT-reducing activity of U-937 cells only marginally. However, when F-DIF was combined with IL-1 and IL-4,

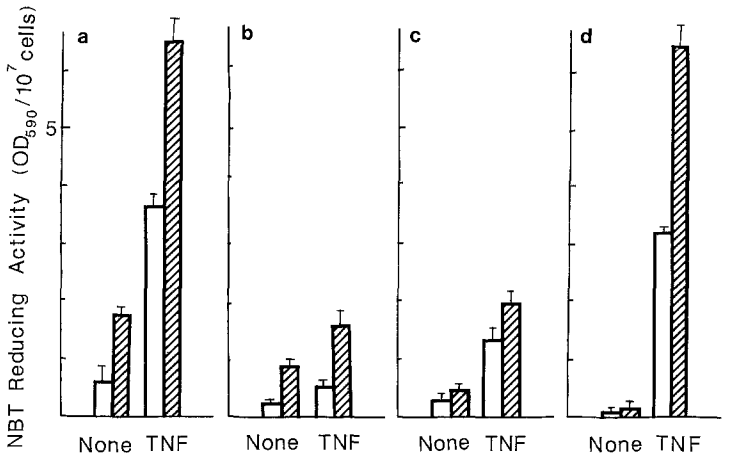

Fig. 1. Effect of F-DIF and TNF on the induction of NBT-reducing activity of various human myelogenous leukemic cell lines. Four cell lines were cultured for 3 days without (□) and with F-DIF 10 ng/ml (▨) either alone or in combination with 1 ng/ml TNF (a, U-937; b, THP-1; c, HL-60; d, ML-1). NBT reducing activity was determined by colorimetric assay described in Materials and Methods. Values are mean of three determinations ± S.E.

it remarkably increased induction of NBT reducing activity. GM-CSF also synergistically induced NBT-reducing activity in combination with F-DIF (Table II).

Table II

Effects of F-DIF in combinations with other cytokines on NBT-reducing activity of U-937 cells

Cytokines		NBT rducing activity ($OD_{590}/10^7$ cells)	
		None	F-DIF(10 ng/ml)
None		0.5 ± 0.1	1.7 ± 0.2
IL-1	1 ng/ml	0.8 ± 0.1	3.7 ± 0.3
IL-2	1 ng/ml	1.0 ± 0.1	2.4 ± 0.2
IL-3	1 ng/ml	0.6 ± 0.1	1.8 ± 0.2
IL-4	1 ng/ml	0.6 ± 0.1	6.7 ± 0.2
G-CSF	1 ng/ml	0.9 ± 0.1	2.6 ± 0.5
GM-CSF	1 ng/ml	1.8 ± 0.1	4.7 ± 0.3
IFN-α	100 U/ml	0.9 ± 0.1	1.4 ± 0.2
IFN-β	100 U/ml	1.1 ± 0.1	2.3 ± 0.5
IFN-γ	100 U/ml	6.5 ± 0.9	8.3 ± 0.4
TNF	1 ng/ml	3.1 ± 0.1	8.6 ± 0.1

U-937 cells were cultured for 3 days with various cytokines at indicated concentrations either alone or in combination with F-DIF 10 ng/ml. NBT-reducing activity was determined by colorimetric assay described in Materials and Methods. Values are mean of three determinations ± S.E.

DISCUSSION

Data presented in this paper show that fibroblast-derived differentiation inducing factor (F-DIF) purified from the culture medium of a human fibroblast cell line WI-26VA4 is able to induce the differentiation of the human monoblastic leukemia cell line, U-937, into cells with macrophage characteristics. F-DIF also induced the differentiation of another human monoblastic leukemia cell line, THP-1. On the other hand, F-DIF induced the differentiation of ML-1 (a human myeloblastic leukemia cell line) and HL-60 (a promyelocytic leukemia cell line) only marginally, but synergistically increased the differentiation of ML-1 cells when combined with TNF. Some cytokines act synergistically with TNF in the induction of differentiation of U-937 cells. IFN-α and -β, either alone or incombination with F-DIF only marginally induced differentiation, while IFN-γ remarkably induced differentiation either alone or in combination with F-DIF.

IL-1, -2, -3 and -4, alone, had almost no differentiation inducing activity against U-937 cells. However, when IL-1, a T cell activation factor which stimulates both central and peripheral T lymphocytes, and IL-4, a B-cell stimulation factor 1 which induce the differentiation of early stages of B-cells(19), were combined with F-DIF, induction of NBT reducing activity was synergistically increased.

The purified F-DIF may be IL-6. Analysis of the 13 N-terminal residues of F-DIF indicated a striking homology with the N-terminal sequence of BSF-2 (IL-6) purified from conditioned medium of a T-cell line (21,22). The sequence of N-terminal amino acid residues was the same as that of BSF-2, except for the absence of the N-terminal proline (unpublished data). BSF-2, B-cell differentiation factor, is able to induce the final maturation of B cells into immunoglobulin-secreting cells (23).

Hirano, et al. reported that only human G-CSF among various cytokines showed significant similarity to IL-6 (21). Amino acids 28-91 of IL-6 and amino acids 20-85 of G-CSF match in 17 residues (25.7% of all residues). In addition, cysteine residues at positions 44, 50, 73 and 83 of IL-6 match those at positions 39, 45,, 67 and 77 of G-CSF exactly (21,24).

In the murine system, Tomida, et al. purified a D-factor that induced the differentiation of mouse M_1 cells, but not human myelogenous leukemic cells (25). The molecular weight of the D-factor (62,000) is different from that of F-DIF (27,000) when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

IL-6 induces the differentiation of monocytoid and myeloid leukemic cells. The data suggest that fibroblasts may interact with monocytic cells by producing F-DIF (IL-6), which enhances the differentiation of macrophage progenitor cells, and monocytic cells produce IL-1 and TNF which induce the synthesis of IFN-β_2 (IL-6) in fibroblasts (26,27). These facts indicate that fibroblasts and monocytic cells may interact with each other by producing cytokines such as IL-6, IL-1 and TNF. It is of particular interest that F-DIF (IL-6) in combination with IL-1 or TNF synergistically induced the differentiation of myelogenous leukemic cells.

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