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SYNERGISM OF TUMOR NECROSIS FACTOR AND INTERFERON-γ IN INDUCTION OF DIFFERENTIATION OF HUMAN MYELOBLASTIC LEUKEMIC ML-1 CELLS

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SUMMARY: Natural or recombinant human tumor necrosis factor (TNF) induced NBT-reducing activity of ML-1 cells in a dose-dependent manner. Interferon-γ (IFN-γ) induced NBT-reducing activity only marginally. However, when IFN-γ was combined with TNF, induction of NBT-reducing activity was remarkably increased. IFN-α or -β had almost no effect on the induction of NBT-reducing activity of ML-1 cells, either alone or in combination with TNF. Treatment with both TNF and IFN-γ synergistically enhanced morphological changes, growth inhibition and activity of Fc receptors, and NBT reduction in ML-1 cells, but not phagocytic activity. The TNF treated cells were classified as macrophage-like by morphology, and by lineage-specific α-naphthyl acetate esterase stain. The results indicate that combinations of TNF and IFN-γ act synergestically in the induction of differentiation of human myeloblastic ML-1 cells.

Human myeloid leukemia cell lines or cells derived from patients can be induced to differentiate into the monocyte/macrophage pathway by protein inducers prepared from the conditioned media of mitogen-stimulated human leukocytes (1-4).

Differentiation inducing factor (DIF) has been partially purified from medium conditioned by phytohemagglutinin-stimulated lymphocytes (5) or T cells (6). These factors differed in activity from colony-stimulating factors or interferons (IFNs) (5,6).

Abbreviations: TNF, tumor necrosis factor; IFN, interferon; NBT, nitroblue tetrazolium; LCM, leukocyte conditioned medium.

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We demonstrated previously that IFN-γ, purified from PHA-stimulated human leukocyte conditioned medium (LCM), could be both an inducer and an enhancer of induction of human myelogenous leukemia cells. At the same time, even if IFN-γ was eliminated from LCM by affinity chromatography, the LCM had differentiation inducing activity (7).

Recently we succeeded in purifying a DIF from LCM from which IFN-γ had been eliminated. Analysis of the N-terminal amino acid sequence showed that the purifed DIF was a tumor necrosis factor (TNF) (8). TNF was so named because this molecule induced hemorrhagic necrosis in certain animal tumors and in some heterotransplanted human tumors (9,10). It also exhibited cytostatic and cytolytic activity against transformed cell lines in culture (9-12).

On the other hand, IFNs have been divided into three major classes, IFN-α, IFN-β, and IFN-γ, based on antigenic and physicochemical properties (13). These INFs are shown to have multiple biological functions, including antiviral, antitumor, and immunomodulatory activity as well as ability to modify the differentiation program of both normal and transformed cells (14-16).

In the present study, we examined the effects of combinations of TNF and IFNs (α, β and γ) on the induction of differentiation of the human myeloblastic leukemia cell line, ML-1.

MATERIALS AND METHODS

RPMI 1640 medium was purchased from Grand Island Biological CO., Grand Island, New York, and fetal bovine serum was obtained from Filtron Pty. Ltd., Victoria, Australia. Nitroblue tetrazolium dye was purchased from Sigma Chemical Co., and 12-O-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Chemicals for Cancer Research, Inc, Eden Prairie, Minn.

Highly purified natural and recombinant IFNs were kindly donated by The Green Cross Co., Osaka, Shionogi Pharmacentical Co., Osaka and Toray Co., Tokyo,Japan.

TNF was purifed to homogeneity from a medium conditioned by phytohemagglutinin stimulated human peripheral blood mononuclear

cells. Recombinant human TNF was kindly donated by Dainippon Pharmaceutical Co., Osaka, Japan.

Cell culture: Ml-l cells, established from a patient with human acute myeloblastic leukemia (17), 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 $6x10^4$ cells, 10% FBS and test materials at the desired concentrations.

The effects of the TNF and/or IFN-γ 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 cellar markers normally associated with maturation of the monocytic elements.

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 (18).

NBT reducing ability: NBT-reducing activity was assayed by the modified method of Baehner and Nathan (19); $3x10^5$ 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 40 minutes at 37°C in a 5% CO_2 incubator.

Microscopic assay: The percentage of cells containing blue-black formazan deposits was determined by counting at least 200 cells.

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.

Phagocytic activity: Phagocytic activity was determined by the method of baker's yeast digestion (20). 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 ML-1 cells that digested yeast particles were scored as phagocytosis positive, and at least 200 viable cells were counted in each determination.

Morphological differentiation: The appearance of morphological differentiation was assessed in stained slide preparations. Slides were prepared by centrifugation in a Cytospin, Shandon Southern Products, Ltd., U.K. 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 chloroacetate esterase and α-naphthyl acetate esterase activity was performed as previously described (21).

RESULTS

Dose-dependent effect of TNF on induction of NBT-reducing activity of ML-1 cells in the presence or absence of IFN-γ.

Natural TNF, purified from PHA-stimulated human leukocyte conditioned medium, induced NBT-reducing activity of ML-1 cells,

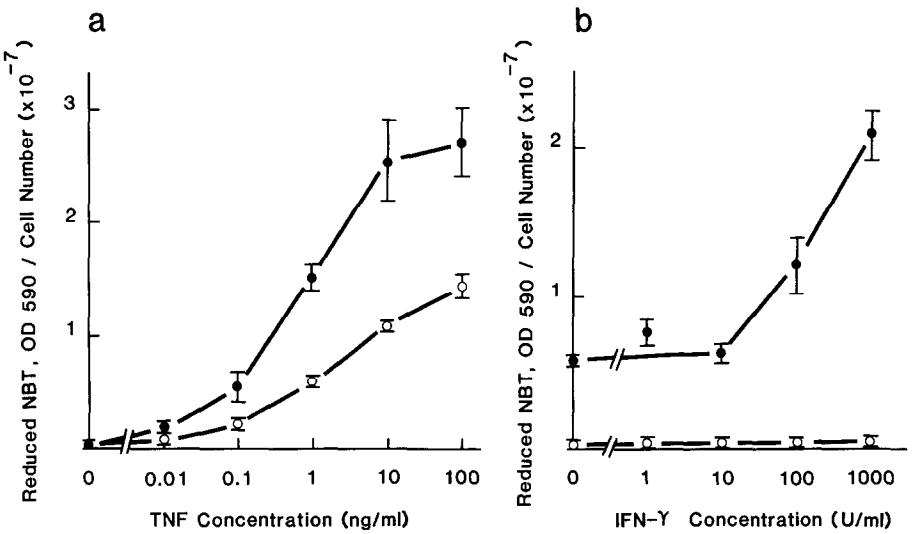

Fig. 1. Dose-dependent effects of combinations of TNF and IFN-γ on induction of differentiation of ML-1 cells. a: ML-1 cells were cultured for 3 days with TNF at indicated concentrations in the presence (•) or absence (o) of IFN-γ (100 units/ml). b: ML-1 cells were cultured for 3 days with IFN-γ at indicated concentrations in the presence (•) or absence (o) of TNF (1ng/ml). NBT-reducing activity was determined by colorimetric assay described in Materials and Methods. Values are mean of three determinations ± S.E. Separate experiments varied by ≤ 20%.

dose dependently. TNF was less cytotoxic against ML-1 cells. Highly purified natural human IFN-γ alone induced almost no NBT-reducing activity at a concentration of 100 units/ml, but when 100 units/ml IFN-γ was combined with TNF, induction of NBT-reducing activity was remarkably increased (Fig.1a). Similar results were obtained using recombinant human TNF and IFN-γ (not shown).

Dose-dependent effect of IFN-γ on induction of NBT-reducing activity of ML-1 cells in the presence or absence of TNF.

As shown in Fig. 1b, IFN-γ did not induce NBT reducing activity of ML-1 cells up to a concentration of 1,000 units/ml. However, in the presence of 0.1 ng/ml TNF it enhanced the NBT-reducing activity, dose-dependently, at concentrations above 10 units/ml.

Table 1. Comparison of TNF combinations with human IFN-α, -β and -γ in induction of NBT-reducing activity of ML-1 cells. ML-1 cells were cultured for 3 days with IFN-α, -β and -γ at a concentration of 1,000 units/ml either alone or in combination with TNF 0.1 ng/ml. NBT reducing activity was assayed by microscopy and growth inhibition was determined by counting cell numbers with hematocytometer as described in Materials and Methods. Values are mean of three determinations ± S.E. Separate experiments varied by ≦ 20%.

Addition	NBT reducing activity, %		Growth inhibition, %
	in combined with		
	None	TNF 0.1 ng/ml	
None (control)	1±1	15±0	----
IFN-α 1000 U/ml	1±1	14±1	41±9
IFN-β 1000 U/ml	2±1	15±1	51±6
IFN-γ 1000 U/ml	11±2	54±1	38±7

Comparison of human IFN-α, -β, and -γ induction of NBT-reducing activity of ML-1 cells in combination with TNF.

As shown Table 1, IFN-α or -β had almost no effect on induction of the NBT-reducing activity of ML-1 cells at a concentration of 1,000 units/ml, either alone or in combination with 0.1 ng/ml TNF. All interferons, at concentrations of 1,000 units/ml, inhibited cell proliferation 40-50% on day 3.

Combination effects of TNF and IFN-γ on the appearance of differentiation associated characteristics in ML-1 cells.

As shown in Table 2, 1 ng/ml TNF and 100 units/ml IFN-γ, in combination, induced Fc receptor activity and morphological changes synergistically. They also inhibited proliferation synergistically, without significant loss of viability until day 3. However, there was no synergism of phagocytic activity by combination of both agents. The TNF and IFN-γ treated cells were classified morphologically as macrophage-like cells that had increased cytoplasm, abundant granules and an eccentrically placed oblate nucleus with loosely stranded nuclear chromatin. The activity of macropage-specific α-naphthyl acetate esterase was increased (data not shown).

Table 2. Effects of combinations of TNF and IFN-γ on the appearance of differentiation-associated characteristics of ML-1 cells. ML-1 cells were cultured with 1ng/ml TNF and 100 units/ml IFN-γ , either alone or in combination. Differentiation was monitored by determining the appearance and accrual of various cellular markers normally associated with maturation of the monocytic elements. Fc receptor activity was measured on day 1. NBT-reducing activity, phagocytic activity and the rate of growth inhibition were determined on day three. Morphological differentiation was assayed on day six. Values are mean of three determinations ± S.E. Separate experiments varied by ≦ 20% .

Addition	Fc receptor (%)	NBT reduction (%)	Phago-cytosis (%)	Maturing cells (%)	Growth inhibition (%)
None (control)	7±2	1±1	2±1	3±1	----
TNF 1ng/ml	11±2	35±2	62±2	20±1	29±4
IFN-γ 100U/ml	29±4	1±1	9±1	9±1	18±6
TNF 1ng/ml+IFN-γ 1 100U/ml	47±3	91±2	61±2	65±8	100±0

DISCUSSION

The present study demonstrated that TNF and IFN-γ in combination synergistically induced differentiation of the human myeloblastic leukemia cell line, ML-1, into cells with macrophage characteristics. TNF has been found to be specifically toxic to many tumor cells (9-12). However, ML-1 was rather resistant to the cytotoxicity of TNF, which induced differentiation of ML-1 cells, dose-dependently. On the other hand, IFN-γ induced differentiation only marginally, but synergistically increased differentiation when combined with TNF. The mechanism of the synergism of TNF and IFN-γ in induction of differentiation are unknown.

Recently Aggarwal *et al*. showed that IFN-γ increased the total number of TNF receptors two to three fold with no significant change in the affinity constant in the human cervical carcinoma cell line (22). Similar phenomena were also observed by Tsujimoto *et al*. (23). They suggested that induction of TNF receptors might contribute to the enhancement of TNF cytotoxicity

seen in some tumor cell lines after IFN treatment. Therefore, study of ML-1 receptors for both TNF and IFN-γ are currently under way in our laboratory.

IFN-α and -β were impotent in inducing differentiation of ML-1 cells, either alone or in combination with TNF. Since IFN-α and -β inhibited cell proliferation of ML-1 cells, as did IFN-γ , the ability of IFN-γ to induce differentiation synergistically with TNF may not be due to its inhibition of cell proliferation.

Synergistic effects of TNF and IFN-γ were observed in several markers associated with differentiation of ML-1 cells, but not in phagocytic activity. The reason why synergism of TNF and IFN-γ does not extend to phagocytic activity is unknown. Degre et al. reported that treatment of mouse peritoneal macrophages with IFN-γ depressed phagocytic activity. This was in contrast to the effect of similar concentrations of α and β interferons, which stimulated phagocytic activity (24). At the present time we have no information regarding the mechanisms of the divergence of these activities.

Our findings suggest that TNF and IFN-γ may be important in the host defense system by modulating maturation of macrophage precursor cells. The synergism between TNF and IFN-γ in induction of differentiation is of considerable interest, because it may provide a way to control terminal differentiaton of clinical leukemia.

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