Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 379-388

RECOMBINANT HUMAN INTERFERON SENSITIZES RESISTANT MYELOID LEUKEMIC CELLS TO INDUCTION OF TERMINAL DIFFERENTIATION

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Received June 3, 1985

SUMMARY: Recombinant human leukocyte interferon (IFN-αA) inhibits growth of the human promyelocytic leukemic cell line HL-60 without inducing these cells to differentiate terminally. When IFN-αA is combined with agents capable of inducing differentiation in HL-60 cells, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), cis or trans retinoic acid (RA) or dimethylsulfoxide (DMSO), growth suppression and induction of differentiation are dramatically increased. By growing HL-60 cells in increasing concentrations of TPA, RA, or DMSO, a series of sublines have been developed which are resistant to the usual growth inhibition and induction of differentiation seen when wild type HL-60 cells are exposed to these agents. Treatment of these resistant HL-60 cells with the combination of IFN-αA and the appropriate inducer results, however, in a synergistic suppression in cell growth and a concomitant induction of terminal differentiation. The ability of interferon to interact synergistically with agents which promote leukemic cell maturation may represent a novel means of reducing resistant leukemic cell populations.

The interferons represent a family of proteins with antiviral, antiproliferative and immunomodulatory activities (1-4). Recent advances in recombinant DNA technology have made highly purified interferon preparations available (5,6), and clinical trials employing recombinant leukocyte interferon (IFN-α) have recently been initiated in cancer patients (7-9). Among its numerous cell modulating activities, interferon can modify the

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ABBREVIATIONS USED: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; RA, 12, 13-cis or 12, 13-trans retinoic acid; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; NBT, nitroblue tetrazolium.

0006-291X/85 $1.50

differentiation program of both normal and transformed cells in culture (10,11). Interferon inhibits adipocyte formation in mouse 3T3 cells (12,13), melanogenesis in B-16 mouse melanoma cells (14,15) and erythrogenesis in Friend erythroleukemia cells (16,17). In contrast, interferon enhances the maturation of normal human skeletal myoblasts (18), human melanoma cells (19), a human monoblastoid cell line U937 (20) and mouse myeloid leukemic cells (21). It has also been postulated that interferon may account for the ability of lymphokine factors to induce differentiation in certain human myeloid and monoblast leukemic cells (20). These observations suggest that in certain cases the inhibition of tumor growth in vivo by interferon may result, at least in part, from a direct modulation of differentiation of the tumor cell (10,11,14-17,19,20).

The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, undergoes terminal differentiation to form either granulocytes or macrophages in the presence of 12-O-tetradecanoyl-phorbol-13-acetate (TPA)(22-24), 12,13-cis or 12,13-trans retinoic acid (25) or dimethylsulfoxide (DMSO) (26). The mechanism by which these agents exert their effects is not known, but may involve more than one locus of action. For example, TPA and related phorbol ester tumor promoters bind to high affinity membrane-associated and cytosolic receptors, which appear to be the enzyme protein kinase C (27-29). In contrast, the retinoids may act through specific receptors present in the cytosol (30,31). Purified preparations of leukocyte and fibroblast interferon inhibit the growth of HL-60 cells, but do not induce them to differentiate terminally (32). However, recent studies suggest that both leukocyte and fibroblast interferon can enhance the response of these cells to either TPA or 12,13-trans RA (33). In contrast, immune interferon (IFN-γ) has been shown by Ball et al. (34) to induce in some HL-60 populations the expression of antigens characteristic of monocytes and granulocytes, as well as morphological alterations characteristic of monocytoid differentiation. By growing HL-60 cells in progressively higher concentrations of TPA, 12-13-trans RA or DMSO we have isolated variants

(designated HL-60/TPA^R, HL-60/RA^R or HL-60/DMSO^R) that continue to grow in the presence of 10^{-7}M TPA, 10^{-6}M 12,13-trans RA or 1.2% DMSO, respectively (35). In contrast, under these conditions the parental cells stop dividing and either die or undergo terminal differentiation. The purpose of the present study was to determine whether interferon might restore the responses of these resistant HL-60 variants to these agents.

MATERIALS AND METHODS

The human promyelocytic leukemia cell line HL-60 was obtained at early passage from the original cell line isolated by Collins, Gallo and Gallegher (26). Cultures were maintained in the logarithmic phase of growth in RPMI medium supplemented with 1% sodium pyruvate, nonessential amino acids and 10% heat inactivated fetal bovine serum (GIBCO). Cells were subcultured twice weekly in 75 cm^2 sterile plastic tissue culture flasks (Corning) and maintained in a fully-humidified 37°C, 5% CO_2 water-jacketed incubator (Napco). Resistant HL-60 variants were produced by initially seeding logarithmically growing HL-60 cells in medium containing 10^{-9}M TPA, 0.7% DMSO or 10^{-8}M trans retinoic acid. After 7 days incubation at 37°C non-adherent cells were layered over a cushion of Ficoll-Hypaque (Sp. grav. 1.077-1.081, Bionetics, Kensington, MD.) and centrifuged for 30 min at 400 x g. The interface layer was washed and cells were resuspended in medium containing the same concentration of inducer. This process was repeated until the growth of cells in the presence of each agent approached that of the untreatred parental HL-60 cells, at which point the concentration of inducer was increased. Thereafter, the concentration of each inducer was increased in gradual increments at approximately 2 week intervals. Cells are currently maintained in medium containing 10^{-8}M TPA, 1.1% DMSO and 10^{-6}M RA, and display greater than 90% viability as determined by trypan blue dye exclusion. The resistance phenotype is a quantitative property of the variants since high levels of the appropriate inducer, i.e. 10^{-6}M TPA, 1.5% DMSO or 5 x 10^{-5}M RA, will induce growth suppression and differentiation in the resistant variants.

Recombinant leukocyte interferon (IFN-αA) was prepared as previously described (36). The interferon preparations were stored in sterile 2 ml vials at -80°C, thawed immediately prior to use and diluted to the appropriate concentration in RPMI medium. TPA, DMSO and 12-13-trans RA were purchased from Sigma, St. Louis, Mo. Drugs were stored in the dark at -20°C and reconstituted in RPMI medium in subdued light immediately prior to each experiment. For suspension culture growth experiments, 5 ml of cells were seeded into 35 mm six well plates (Costar) at an initial density of 10^5 cells/ml. TPA, RA, DMSO and IFN-αA, alone or in combination, were added to the cells at the designated concentrations and the plates were incubated at 37°C. Aliquots of 0.5 ml were removed at 24 hr intervals and cell number determinations were obtained with a Model ZBI Coulter Counter (Hialeah, Fla.). After 5 days growth, the cells were pelleted and cytocentrifuge preparations were made with a Shandon cytocentrifuge. Slides were then air dried and stained with Wright-Giemsa. For nitroblue tetrazolium (NBT) dye reduction studies, 1 ml of cells suspended at 2 x 10^6 cells/ml in RPMI medium supplemented with 20% FBS was incubated for 20 min at 37°C with an equal volume of 0.2% NBT (Sigma) in the presence of 200 ng of TPA. The percent of cells containing intracellular reduced blue-black formazan deposits was then determined on Wright-Giemsa stains of cytospin preparations of incubated cells.

The ability of sensitive and resistant HL-60 cells to form colonies in soft agar in the presence of TPA, DMSO, RA or IFN-αA, alone and in various combinations, was determined by a minor modification of a previously described technique (37, 38). Cells were seeded in 18 mm 12 well dishes (Costar) utilizing a two layer agar system (38). The bottom layer consisted of 0.5 ml of RPMI medium supplemented with 1% sodium pyruvate, 1% non-essential amino acids, 20% (vol/vol) FBS and 0.5% bacto agar (Difco, Detroit, MI). The top layer, containing the cells, consisted of 0.5 ml RPMI medium, 20% (vol/vol) FBS, 0.3% bacto agar and the appropriate concentration of DMSO, RA or TPA, with or without 1000 I.U./ml of IFN-αA. Each plate contained 2×10^3 sensitive or resistant HL-60 cells and the appropriate additions. After the agar solidified, 0.1 ml of GCT conditioned medium (GIBCO) was added to each plate as a source of colony stimulating activity (39). Cells were grown for 10 days in a fully humidified incubator and colonies, consisting of 50 or more cells, were scored with the aid of an Olympus Model CK inverted microscope.

RESULTS

Figure 1 displays the effects of IFN-αA (1000 I.U./ml) on the growth in suspension culture of the three types of HL-60 variants. When tested alone, IFN-αA produced only a slight growth inhibition, i.e. from 18 to 36% of control. When HL-60/TPA^R, HL-60/RA^R and HL-60/DMSO^R cells were exposed to 10^{-7}M TPA, 2.5×10^{-5}M 12-13-trans RA or 1.2% DMSO, the respective growth inhibition was 23,19 and 40% of control. However, when IFN-αA was combined with the same concentrations of these agents, in all cases there was a greater than 90% inhibition of growth. Similar patterns of response were seen when these agents were studied for effects on colony formation of HL-60 variants in soft agar (Table 1). Again, concomitant exposure of resistant cells to IFN-αA and TPA, RA or DMSO produced a marked synergistic effect with respect to growth inhibition. A synergistic suppression in suspension and agar growth was also observed when sensitive and resistant HL-60 cells were exposed to 250 or 500 I.U./ml of IFN-αA and the appropriate differentiation inducing compound or when recombinant fibroblast interferon was used in place of IFN-αA (unpublished data). In general, the inhibitory effect of interferon as well as TPA, RA or DMSO on the growth of HL-60 cells in soft agar culture was greater than in suspension culture. This difference may indicate a greater susceptibility of subsets of clonogenic cells to the various agents.

To determine whether these antiproliferative effects were associated with enhancement of differentiation, we assessed the ability of treated cells to reduce the dye nitroblue tetrazolium (NBT), a characteristic of mature

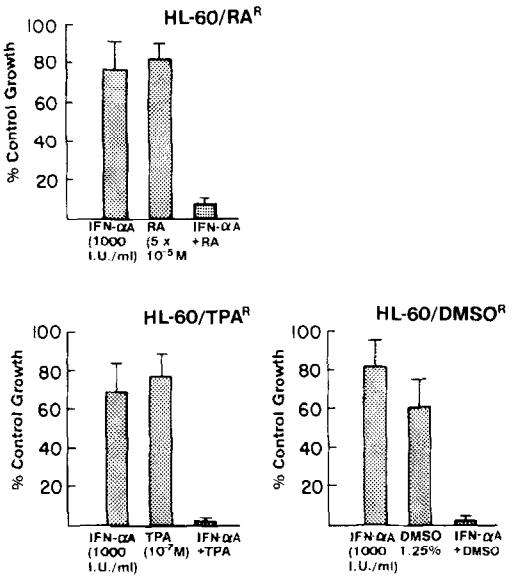

Figure 1

Effects of IFN-αA on the abilities of TPA, 12-13-trans retinoic acid (RA) and DMSO to inhibit the growth of HL60 variants in suspension culture. Cells were suspended in RPMI medium containing 10% fetal calf serum (FCS) at a density of 10^5 cells/ml and placed in 25 cm^2 tissue culture flasks. The indicated concentrations of TPA, RA or DMSO (Sigma Chemicals, St. Louis, MO) were added with and without 1000 I.U./ml IFN-αA. The cells were grown in a 37°C, 5% CO_2 humidified incubator for 72 hr and cell densities were then determined utilizing a Model ZBI Coulter Counter. The heights of the bar graphs represent the percentage of control cell growth for each condition, and represent mean values for at least three separate experiments, each performed in duplicate. The absolute density of the control cultures at 72 hr was $6X10^5$ cells/ml. Not shown are the responses of parent HL-60 cells, which exhibited less than 5% of control cell growth in the presence of the indicated concentrations of TPA, RA or DMSO. Error bars indicate standard deviations between replicate samples. Separate experiments varied by $\leq$ 15%.

neutrophils and macrophages (40). We found that IFN-αA alone (1000 I.U./ml) did not affect this property in the variants (Table II) or in the parental HL-60 cells. However, when the variants were treated with IFN-αA together with TPA, 12,13-trans RA or DMSO there was a dramatic increase in the percentage of cells capable of reducing NBT dye. Interferon also potentiated

TABLE 1

EFFECTS OF IFN-αA IN COMBINATION WITH RA, TPA OR DMSO ON THE GROWTH IN AGAR OF HL-60 DRUG RESISTANT VARIANTS

CELL TYPE	CONDITION	NUMBER OF COLONIES (% OF CONTROL)
HL-60/RAR	IFN-αA (1,000 I.U./ml)	87 ± 13
	RA 10^{-5}M	42 ± 7
	+ IFN-αA (1,000 I.U./ml)	10 ± 2
	RA 2.5 x 10^{-5}M	26 ± 5
	+ IFN-αA (1,000 I.U./ml)	0
HL-60/TPA^R	IFN-αA (1,000 I.U./ml)	80 ± 13
	TPA 10^{-8}M	66 ± 9
	+ IFN-αA (1,000 I.U./ml)	17 ± 4
	TPA 10^{-7}M	49 ± 8
	+ IFN-αA (1,000 I.U./ml)	6 ± 2
HL-60/DMSO^R	IFN-αA (1,000 I.U./ml)	77 ± 12
	DMSO 1%	89 ± 14
	+ IFN-αA (1,000 I.U./ml)	40 ± 6
	DMSO 1.25%	62 ± 9
	+ IFN-αA (1,000 I.U./ml)	11 ± 4

Cells were plated in sterile 18 mm diam. 24 well plates (Costar, Cambridge, MA). The bottom layer consisted of 0.5 ml RPMI medium containing 15% fetal calf serum and 0.5% Noble agar (Difco, Detroit, MI). The top layer contained 2×10^3 cells in 0.5 ml RPMI with 15% fetal calf serum, 0.3% agar, the indicated concentration of TPA, DMSO or RA, with or without 1000 I.U./ml IFN-αA. The plates were placed in a humidified 37°C, 5% CO_2 incubator for 10 days and the numbers of colonies, consisting of groups of 50 or more cells, were scored with the aid of an Olympus Model CK inverted microscope. Values are expressed as the percentage of control colony formation, and represent the means of at least three separate experiments, each performed in duplicate. The values in the table represent the mean ± standard deviation. Not shown are the responses of parental HL-60 cells, which exhibited no colony formation in the presence of the designated concentrations of TPA, RA or DMSO.

TABLE 2

EFFECTS OF IFN-α ON THE ABILITY OF RA, TPA OR DMSO TO INDUCE MATURATION OF SENSITIVE AND RESISTANT HL60 CELLS

CELL TYPE		% NBT+ CELLS IN THE PRESENCE OF:			
		RETINOIC ACID (M)			
HL-60/RAR	-	10^{-7}	10^{-5}	2.5×10^{-5}	5.0×10^{-5}
Control	0	-	2±1	3±2	35±7
IFN-αA 1000 I.U./ml	0	-	38±6	55±9	85±5
HL-60					
Control	0	42±6	-	-	-
IFN-αA 1000 I.U./ml	0	89±6	-	-	-
		TPA (M)			
HL-60/TPAR	-	10^{-9}	10^{-8}	10^{-7}	10^{-6}
Control	0	-	0	2±1	14±4
IFN-αA 1000 I.U./ml	0	-	14±3	30±6	45±7
HL-60					
Control	0	2±1	-	-	-
IFN-αA 1000 I.U./ml	0	19±4	-	-	-
		DMSO (%)			
HL-60/DMSOR	-	0.9	1	1.25	1.5
Control	0	-	2±1	5±2	24±7
IFN-αA 1000 I.U./ml	0	-	9±3	28±6	48±10
HL-60					
Control	0	7±2	-	-	-
IFN-αA 1000 I.U./ml	0	16±5	-	-	-

Cells were plated at 10^5/ml in 25 cm^2 tissue culture flasks in RPMI medium plus 15% FCS containing the indicated concentrations of TPA, RA or DMSO, with and without 1000 I.U./ml IFN-αA. After 7 days at 37°C, in a 5% CO_2 incubator, one ml aliquots of the cell suspensions, containing approximately 10^6 cells, were mixed with an equal volume of 0.2% NBT (nitroblue tetrazolium) dye (Sigma) and 10^{-9}M TPA for 30 minutes at 37°C. The cells were then centrifuged in a Shandon cytocentrifuge at 500 RPM for 5 minutes, fixed on albumin coated microscope slides, and stained with saffranin. The percentage of cells displaying blue-black formazan deposits was determined by counting at least 200 cells. Values represent the means (± S.D.) for at least three separate experiments performed in duplicate.

the effects of lower concentrations of TPA, RA or DMSO on the maturation of parental HL-60 cells in a greater than additive fashion.

DISCUSSION

The results of our present study indicate that leukocyte interferon can restore the ability of a subset of resistant human leukemic cells to respond to inducers of terminal differentiation such as TPA, retinoic acid, and DMSO. Whether this interaction occurs primarily at the level of cell proliferation or differentiation, or possibly both, is at present unclear. For example, it has been demonstrated that two cultured human leukemic cell lines (HL-60 and KG-1) need not undergo cell division in order to differentiate into mature forms in cell culture when stimulated with phorbol esters (41). In addition, although agents which induce leukemic cells to mature will necessarily inhibit their proliferative capacity, the reverse is not true (42). Agents which inhibit cell division may or may not trigger terminal differentiation, depending upon the nature of the agent and its mode of action. It is conceivable that interferon may modulate both the antiproliferative and maturational response of resistant cells to various agents and that these responses may be related but separate phenomena. Moreover, different classes of interferon may have different effects on proliferation and maturation within the same cell system. As discussed previously, leukocyte and fibroblast interferons primarily inhibit HL-60 proliferation, whereas immune interferon may both inhibit proliferation and induce differentiation in some HL-60 populations. Finally, it is of interest that interferon was able to potentiate the activity of three quite different classes of agents, which presumably influence differentiation through different mechanisms. We doubt that this effect is simply at the level of initial receptor binding since it is known that HL-60/TPA^R cells isolated by others retain apparently normal levels of phorbol ester receptors (43,44). In view of the evidence for membrane-associated receptors for interferon (45-52), it is possible that IFN-αA sensitizes these variants through a more generalized change in cell membranes. In separate studies it has been found that IFN-αA or recombinant

fibroblast interferon acts synergistically with the compound mezerein to inhibit growth and induce differentiation in certain human melanoma cell lines (10,14). This synergistic response occurs even in melanoma cell lines relatively resistant to the growth suppressing and differentiation inducing effect of interferon or mezerein when applied alone (19). It will also be of interest, therefore, to determine whether various types of interferons are capable of facilitating terminal differentiation in drug resistant tumor systems, and whether this combined approach to treatment might increase the clinical effectiveness of interferon as an antitumor agent.

ACKNOWLEDGEMENTS: This work was supported by an award from Hoffmann-La Roche Inc. to P.B.F. and I.B.W. We thank Barbara Hamilton for assistance in the preparation of this manuscript.

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