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INTERFERON INHIBITS MELANOGENESIS IN B-16 MOUSE MELANOMA CELLS

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SUMMARY: Mouse L-cell interferon (IF) (0.03-30 units/ml) produces a concentration-dependent inhibition of both spontaneous and melanocyte hormone stimulated (MSH) melanogenesis in cultures of murine B-16 melanoma cells. This inhibition is synergistic with that produced by the tumor promoter 12-O-tetradecanoyl-phorbol-13 acetate (TPA). Both TPA and IF also inhibit the expression of tyrosinase activity. These and previous results suggest that the antitumor effects of IF may be due to modulation of cellular differentiation.

INTRODUCTION

In addition to its antiviral (1) and antitumor (2) effects, interferon (IF) is a potent modulator of cellular differentiation (1,3-6). Another class of naturally occuring substances, the phorbol ester tumor promoters, also alters the program of differentiation in a wide variety of cell culture systems (for review see 7,8). We have previously demonstrated that the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) can delay both spontaneous and MSH-induced differentiation of the C_3 clone of B-16 melanoma cells (8). In addition, we found that the naturally occurring membrane active peptide melittin also delays the expression of melanogenesis in B-16 cells (9). The primary site of action for interferon (1,10), TPA (7) and melittin (9) appears to be the cell membrane (1,7,9,10). For these reasons, in the present study we have examined the effects of interferon, alone or in combination with phorbol esters, on differentiation of the B-16 melanoma cells.

Abbreviations used: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; MSH, α-melanocyte-stimulating hormone; IF, mouse L-cell interferon; PDD, phorbol-12, 13-didecanoate; 4αPDD, 4α-phorbol-12,13-didecanoate; DMSO, dimethylsulfoxide; PBS, phosphate buffered saline.

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MATERIALS AND METHODS

Cell Culture: The C_3 clone of B-16 mouse melanoma cells (11) was provided by Dr. John Kreider of the Hershey Medical Center, Hershey, Pa. Cells were routinely maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, New York) and 2 mM L-tyrosine. Cells were passaged as previously described (8) using 0.2% versene in phosphate-buffered saline (PBS) (0.01 M phosphate pH 7.4 and 0.15 M NaCl).

Experimental Design: B-16 cells were plated at $5x10^5$ cells/5cm dish. Compounds to be tested, unless otherwise indicated, were added at the time of cell plating in a volume of DMSO such that the final solvent concentration did not exceed 0.01%. Cell counts were determined by removing cells from plates with 0.2% versene in PBS, diluting cell suspensions with isotonic diluent (Fisher Scientific Co., Fairlawn, New Jersey) and counted using an electronic model Z_f Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.).
Melanin Determinations: The melanin content of the cells and the cell culture medium was determined as previously described by Mufson et al. (8).
Tyrosinase Determinations: The tyrosinase activity of the cells was determined by a modification of the Pomerantz radioassay described by Mufson (12).
Protein Determinations: Protein content of the cells was determined according to the method of Lowry.
Mouse L-Cell Interferon: Mouse interferon was prepared from L-cells grown in monolayer culture in Eagle's minimal essential medium containing gentamicin (50 µg/ml) and 10% fetal bovine serum. Induction of interferon was performed as described (13-15) with the substitution of poly(I)poly(C) (50 µg/ml) for Newcastle disease or MM virus. Assay of mouse interferon was performed by a cytopathic effect-inhibition assay with L-cells and vesicular stomatitis virus. Units of interferon were adjusted to the international reference standard for mouse interferon (G-002-904-511) supplied by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

RESULTS

Addition of 0.03 to 30 units/ml of mouse L-cell interferon (IF) to B-16 cells at the time of plating produced a dose-dependent inhibition of melanin production when the latter was measured on day 6, the time when the control cells displayed extensive melanogenesis (Table 1). In contrast, human leukocyte IF (100 units/ml) and heat treated (60 min. at 60°C) or trypsin treated (5mg/ml for 30 min.) mouse L-cell IF did not inhibit melanogenesis by B-16 cultures. The inhibitory effect of 30 IF units/ml was not a result of inhibition of cell growth or the time at which B-16 cells became confluent (data not shown). The suppression of melanogenesis obtained with 30 units/ml of mouse IF was quantitatively similar to that observed when B-16 cells were treated with 100 ng/ml of TPA (Table 1).

Table 1: The Effect of Interferon (IF) Alone or in Combination with TPA on Melanin Synthesis by B-16 Cells.

	TOTAL MELANIN CONTENT (% of Control)			
	Day 6		Day 7	
Compound	Alone	+IF	Alone	+IF
Media Control	100	10	100	103
0.01% DMSO	100	14	100	98
TPA	15	10	104	25
PDD	16	12	110	22
Phorbol	102	15	100	106
4αPDD	109	12	99	108
IF	10	—	105	—

The indicated compounds were added alone or in combination with IF at the time of plating 5 x 10^5 cells/5 cm plate. Where indicated IF was added at 30 units/ml and the other compounds at 100 ng/ml. Six and 7 days post-plating cell number and total melanin content (cells plus medium) of the cultures were determined, as described in "Materials and Methods". The melanin content at day 6 and 7 is expressed as a percentage of that found in the DMSO controls. The total melanin content of the DMSO control cultures at day 6 was 4.5 ± 0.52 A_{400} units/10^6 cells and at day 7 was 7.3 ± 0.96 A_{400} units/10^6 cells. Values are the means of triplicate cultures. The S.E. for triplicates was ≤ 20% of the mean.

As previously reported for B-16 cells treated with TPA (8), the cultures treated with IF escaped the inhibition of differentiation at about 24 hours after the control cultures had differentiated (Table 1). If, however, TPA (100 ng/ml) was added together with increasing concentrations of mouse L-cell IF the inhibition of differentiation was sustained longer than with either TPA or IF alone (Table 1). The rapidity of escape was inversely proportional to the concentration of IF. Phorbol compounds structurally related to TPA, but inactive as tumor promoters on mouse skin (7), such as phorbol and 4α phorbol-12,13- didecanoate (4α PDD), did not inhibit B-16 differentiation when added alone or in combination with 30 units/ml of IF. The active tumor promoter PDD, however, did inhibit melanogenesis when added alone and further enhanced IF's inhibitory action (Table 2). TPA and IF also act synergistically to inhibit adipocyte differentiation by Balb/c 3T3 cells (4).

In previous studies (8) we demonstrated that the degree of inhibition of melanogenesis by TPA in B-16 cells was related to its time of addition.

Table 2: The Effect of Phorbol Esters Alone or in Combination With Interferon (IF) on Melanin Synthesis by B-16 Cells.

		TOTAL MELANIN CONTENT (% of Control)*	
Compound	Concentration	Day 6	Day 7
DMSO	0.01%	100	106
IF	0.03 units/ml	82	105
IF	0.3 units/ml	66	99
IF	3 units/ml	43	110
IF	30 units/ml	19	103
TPA	100 ng/ml	21	100
IF + TPA	0.03 units/ml + 100 ng/ml	18	89
IF + TPA	0.3 units/ml + 100 ng/ml	20	68
IF + TPA	3 units/ml + 100 ng/ml	17	48
IF + TPA	30 units/ml + 100 ng/ml	19	27

*The indicated compounds were added at the time of plating 5 x 10^5 cells/5 cm plate. Six and 7 days post-plating cell number and total melanin content (cells plus medium) of the cultures were determined, as described in "Materials and Methods". The melanin content at day 6 is expressed as a percentage of that found in the DMSO control. The melanin content at day 7 is expressed as a percentage of that found in the TPA treated cultures which had terminally differentiated. The total melanin content of the 0.01% DMSO cultures at day 6 was 4.0 $\pm$ 0.36 A_{400} units/10^6 cells; in the 100 ng/ml TPA cultures at day 7 this value was 6.2 $\pm$ 0.85 A_{400} units/10^6 cells. Values represent the means of triplicate cultures, the S.E. for triplicates was $\leq$ 20% of the means.

Marked inhibition was obtained when TPA was added shortly after plating the cells; if the addition of TPA was delayed to days 4 or 5 the cells were much more resistant to inhibition of melanogenesis. Table 3 indicates that IF (30 units/ml) also exerted its maximum inhibition when added during the first few days after

Table 3: The Effect of Time of Addition of Interferon or TPA on Melanin Synthesis by B-16 Cells.

	TOTAL MELANIN CONTENT (% of Control)			
	+TPA (100 ng/ml)		+IF (30 units/ml)	
	Day 6	Day 7	Day 6	Day 7
Time of Addition (day)				
3	16	40	14	45
4	27	61	15	44
5	117	110	79	105

Five x 10^5 cells/5cm plate were seeded at day 0 and 3,4 or 5 days post-plating the medium was removed, TPA or IF was added to this conditioned medium and the medium replated onto the cells. The total melanin content at day 6 and 7 is expressed as a percentage of the 0.01% DMSO control, which was indistinguishable from media controls. The total melanin content of the DMSO control at day 6 was 4.2 $\pm$ 0.47 A_{400} units/10^6 cells and at day 7 was 8.1 $\pm$ 0.85 A_{400} units/10^6 cells. Values are means of triplicate cultures. The S.E. for triplicates was $\leq$ 20% of the mean.

Table 4: The Effect of Interferon (IF) Alone and in Combination With TPA on MSH-Induced Melanogenesis in Clone C_3 of B-16 Cells.

	TOTAL MELANIN CONTENT (% of Control)		
	Day 5	Day 6	Day 7
Compound			
MSH	100	100	100
MSH + IF	13	41	78
MSH + TPA	43	81	84
MSH + IF + TPA	1	36	69

The indicated compounds were added at the time of plating 5 x 10^5 cells/5cm plate, at the following concentrations: MSH 5 x 10^{-7}M, IF 30 units/ml and TPA 100 ng/ml. Five, 6 and 7 days post-plating cell number and total melanin content (cells plus medium) of the cultures were determined, as described in "Materials and Methods". The melanin content at day 5,6 and 7 is expressed as a percentage of that found in the MSH controls. The total melanin content of the MSH control cultures at day 5 was 5.2 ± 0.31 A_{400} units/10^6 cells, at day 6 was 8.4 ± 0.75 A_{400} units/10^6 cells and at day 7, 11.6 ± 1.72 A_{400} units/10^6 cells. The S.E. for triplicate cultures was $\leq$ 20% of the mean.

the cells were plated, even though the control cells did not display an increase in melanin synthesis or tyrosinase activity (see below) until day 5-6. These results suggest that both TPA and IF inhibit a stage which precedes melanin synthesis rather than the actual process of melanogenesis.

In addition to inhibiting spontaneous melanogenesis, both TPA and IF were capable of inhibiting MSH-induced melanogenesis in B-16 cells (Table 4). The addition of 30 units/ml of mouse L-cell IF simultaneously with $5x10^{-7}$ M MSH reduced the amount of pigment produced to $\sim$ 13% of that obtained with cells treated with MSH alone. The inhibition of MSH induced differentiation by IF was greater than the inhibition produced by 100 ng/ml of TPA (43% of control), under comparable conditions. The combination of TPA (100 ng/ml) and mouse IF (30 units/ml) reduced the amount of melanin produced in response to MSH to 1% of that obtained in control cultures exposed only to MSH (Table 4).

To determine whether the delay in onset of melanogenesis induced by IF was due to suppression of actual melanin synthesis, measurements of tyrosinase activity were performed (12). Figure 1 shows that IF (30 units/ml) delayed the peak of tyrosinase activity that occurs when control cells reach confluence.

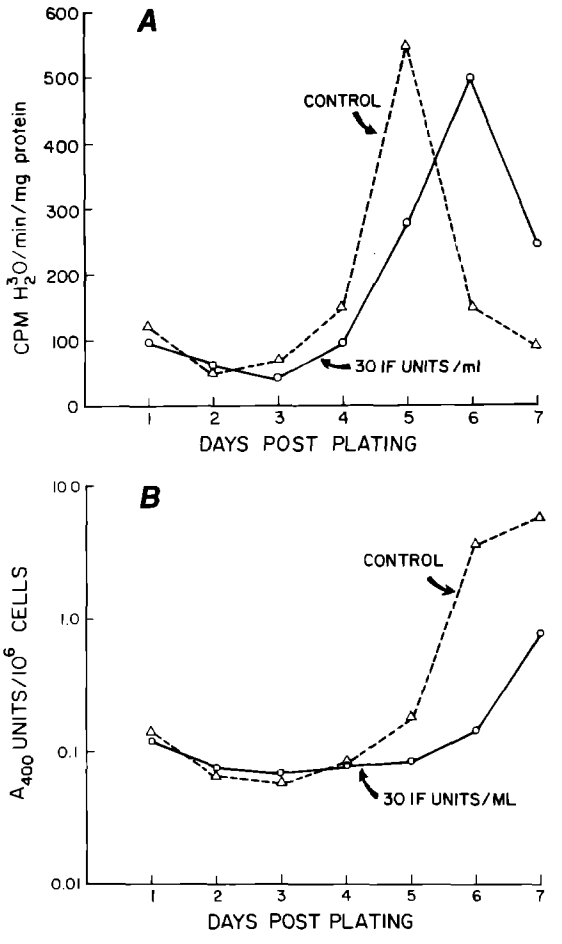

Figure 1: The Effect of IF on Tyrosinase Activity (A) and Melanin Synthesis (B) in B-16 Mouse Melanoma Cells.

Mouse L-cell IF (30 units/ml) was added at the time of plating 5 x 10^5 B-16 cells/5cm tissue culture dish. At the times indicated the cells were collected by scraping with a rubber policeman and tyrosinase activity (A) in the pelleted cells was determined as described in "Materials and Methods". The S.E. for triplicate cultures was $\leq$ 20% of the mean. Melanin content of the cells plus media (B) was determined as described in "Materials and Methods". Values represent the means of triplicate cultures, the S.E. for triplicates was $\leq$ 20% of the mean.

A similar delay in the peak of tyrosinase activity was found with TPA (data not shown).

DISCUSSION

The present results demonstrate that non-cytotoxic doses of mouse L-cell IF suppress both spontaneous and MSH-induced melanogenesis in the C_3 clone

of B-16 mouse melanoma cells. IF also inhibits DMSO induced differentiation in Friend erythroleukemia cells (16,17) and spontaneous adipocyte conversion of 3T3 cells (4,5). In mouse myeloid leukemia cells, IF exerts an opposite effect, i.e., it enhances the differentiation induced by differentiation stimulating factor, lipopolysaccharide or polyinosinic acid (6). Phorbol ester tumor promoters can also have reciprocal effects on differentiation depending upon the cell systems studies (7,18,19). A primary site of action for both IF (1,10) and TPA (7) appears to be the cell surface membrane. However, since IF and TPA act synergistically to inhibit melanogenesis (present studies) and adipocyte formation (5), it appears that they modulate differentiation through somewhat different mechanisms. We have also obtained evidence that they do not occupy the same cell surface receptors (Ivanovic, V., Fisher, P.B. and Weinstein, I.B., unpublished studies).

The antitumor effects of IF have been attributed to either: 1) enhancement of immune defense mechanisms in the host (1) or 2) a direct inhibition of growth of the tumor cells (2,20-22). We suggest that in some cases the antitumor effects may be due to modulation of the differentiated state of the tumor cells. Thus, further studies of the effects of various preparations of IF on differentiation might provide important insights into their more effective use as antitumor agents.

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