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Opposing effects of interferon produced in bacteria and of tumor promoters on myogenesis in human myoblast cultures

(differentiation/creatine kinase/myotubes/recombinant gene product)

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ABSTRACT **We have studied the effects of human leukocyte interferon produced in bacteria and diterpene phorbol ester tumor promoters on differentiation of normal human myoblast cultures derived from mature skeletal muscle. Interferon (100–5,000 units/ml) induced an acceleration of myotube formation and creatine kinase (CK; EC 2.7.3.2) isoenzyme transition from CK-BB to CK-MM. Heat-inactivated or trypsin-treated interferon did not affect the differentiation process. In contrast, the potent tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA), but not its inactive structural analogues phorbol and 4α-phorbol 12,13-didecanoate, caused a dose-dependent (0.01–100 ng/ml) inhibition of myotube formation and CK isoenzyme transition. Neither interferon nor TPA had a significant effect on myoblast proliferation prior to fusion, and the cloning efficiencies were similar as well. Opposing effects of interferon and TPA were also demonstrated by simultaneous application of these agents to the cultures. These studies suggest that some of the antitumor effects of interferon may relate to its capacity to modulate cellular differentiation.**

Several studies have indicated that various preparations of interferon (IFN) can inhibit the development and growth of specific tumors *in vivo* (for review, see refs. 1 and 2). These effects are often explained by the apparent ability of IFN to augment the immune defense mechanism of the host (1–3). On the other hand, there is no direct evidence that the latter effect is sufficient to explain the antitumor effect. In addition, it is clear from *in vitro* studies that IFN can have a direct inhibitory effect on the proliferation of certain cells in culture, in the absence of the immune system (1–6). There is accumulating evidence that IFN can modulate the program of differentiation of several types of tumor cells *in vitro* (7–12). We think it is likely that, at least in certain cases, the antitumor activity of IFN relates to the latter effects, perhaps because, in certain tumors, IFN can induce changes in the program of cellular differentiation that limit the growth potential of the tumor cells and in some cases actually can cause terminal differentiation.

Another class of naturally occurring products, the phorbol ester tumor promoters, are also potent modulators of cellular differentiation, either inhibiting (13–17) or inducing (17–20) differentiation, depending on the target cell. Recent studies indicate that both IFN and the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) can exert synergistic effects in inhibiting (7, 11) or inducing (12) differentiation.

In view of these considerations, in the present study we analyzed the effects of IFN preparations produced in bacteria and of phorbol esters, applied alone or in combination, on the program of differentiation in a human cell culture system. Recent studies have led to the development of highly reproducible methods for the *in vitro* growth of myogenic muscle satellite cells obtained from normal adult human skeletal muscle (21, 22). These cultures recapitulate normal myogenesis, a process that can be followed with specific morphologic and biochemical markers and thus provides a useful system for assessing the effects of various agents on the process of cellular differentiation in human cells.

MATERIALS AND METHODS

Culture Systems. *Mass cultures.* The muscle cultures were grown from human skeletal muscle biopsy specimens obtained for diagnostic evaluation (from patients of either sex and ranging from 8 months to 46 years in age), with advised consent, which were deemed to be free of muscle disease by established histochemical and biochemical criteria. The cultures were first grown as described (21), trypsinized, and plated for 15–20 min to remove the adherent fibroblast contaminants (21, 23). The myoblast-rich supernatant was transferred to culture flasks (Falcon) and grown in Eagle minimal essential medium supplemented with nonessential amino acids, vitamins, sodium pyruvate, and 15% pre-tested fetal bovine serum (GIBCO). Penicillin (75 units/ml) and streptomycin (75 μg/ml) were also added. For growth curves, the preplated (21, 23) cell samples were seeded in 35-mm 6-pak Costar cluster dishes at 5×10^4 or 7.5×10^4 cells per dish. They were left to recover overnight (16–18 hr) and mock-treated with 0.01% dimethylsulfoxide, interferon, phorbol, or phorbol esters at concentrations indicated in the text. The media were removed and cultures were re-fed with control or drug-containing medium (1 ml per dish) every 48 hr; cells were counted in a hemocytometer every 48 hr.

Clonal cultures. Clonal cultures were grown from cell preparations similar to those described above but seeded in 100-mm Falcon culture dishes at 200 or 400 cells per dish in 4 ml of medium. These were left undisturbed for 48 hr. Drug-containing medium was then applied every 72 hr by adding appropriate drug concentrations in "conditioned medium"—i.e., medium previously exposed for 24 hr to mass monolayer cultures and filtered through 0.22-μm-pore Nalgene sterile filters. Cloned cultures were fixed after 14 and 21 days and the colonies were counted. All cultures were grown in an incubator at 36.5°C, in 5% CO_2/95% air kept at saturation humidity.

Fusion Index. The degree of fusion was monitored in replicate cultures grown on coverslips or as clones in 100-mm dishes after the cells were fixed for 10 min with 100% ethanol and

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Abbreviations: IFN, interferon; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; CK, creatine kinase; 4α-PDD, 4α-phorbol didecanoate; 4-*O*-MeTPA, 4-*O*-methyl-TPA; IFLrA, recombinant human leukocyte IFN produced in bacteria; PDB, phorbol 12,13-dibenzoate; PDD, phorbol didecanoate.

stained with 1:10 Giemsa solution (GIBCO) at pH 6.8 in phosphate-buffered saline for 10–15 min. The degree of fusion in mass cultures was determined from the percentage of total nuclei that were incorporated in tri- or multinucleated myotubes. At least 2,000 nuclei were counted in each preparation. Fields were chosen randomly with an ocular grid and ×20 microscopic magnification. The total number of clones per dish were also counted, and the percentage of clones that showed fusion was determined in triplicate.

Creatine Kinase. For creatine kinase (CK; EC 2.7.3.2) measurements, cells were harvested by trypsinization, treated with growth medium containing 20% fetal bovine serum to stop trypsin activity, and washed three times with cold phosphate-buffered saline (pH 7.4) and pelleted by centrifugation at 2,000 × *g*. The pellets were ultrasonicated on ice twice for 15 sec in 50 or 150 μl of phosphate-buffered saline and centrifuged at 4°C for 15 min at 10,000 × *g*. Total CK activity was determined in the supernates by the method of Oliver as modified by Rosalki (24); Caltech CK reagents, to which 25 μM of adenosine dipentaphosphate (Sigma) was added to inhibit adenylate kinase activity, were used (21). Proteins were measured according to Lowry (25). CK isoenzyme electrophoresis was done in an apparatus (Helena Laboratories, Beaumont, TX) designed for cellulose acetate membranes with a 0.05 M Tris barbital buffer (pH 8.6). Electrophoresis was carried out at 4°C for 15 min at 350 V. The membranes were then removed and sandwiched with a second membrane presoaked for 5 min in 2 ml of CK substrate (Abbott), incubated as described for phosphoglycerate mutase analysis (26), and photographed in the darkroom under ultraviolet light (360 nm) with a Polaroid camera using a yellow filter (2C) and Polaroid 107 film.

Interferons. Crude KG-1 IFN was prepared from the human myeloblast cell line KG-1, as described (27). Recombinant human leukocyte IFN produced in bacteria (called IFLrA) (28) was purified to homogeneity by the use of monoclonal antibodies against leukocyte IFN (29). The IFN titer was determined by inhibition of a cytopathic effect induced by vesicular stomatitis virus on bovine kidney (MDBK) or human fibroblast AG-1732 cells (30). IFN titers are expressed in reference units calibrated against a reference standard for human leukocyte IFN (G-023-901-527) provided by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases (Bethesda, MD).

Phorbol Esters. The phorbol diterpene esters TPA, phorbol 12,13-didecanoate (PDD), 4α-phorbol 12,13-didecanoate (4α-PDD), phorbol 12,13-dibenzoate (PDB), and 4-*O*-methyl-TPA (4-*O*-MeTPA), phorbol, and mezerein were purchased from Consolidated Midland. Stock solutions of 1 mg/ml in dimethyl sulfoxide were divided into small portions and stored at −20°C.

RESULTS

Effects of IFN and Tumor Promoters on Myoblast Growth and Myotube Formation. Continuous exposure of primary normal human myoblast cultures to IFLrA at 1–5,000 units/ml or to crude KG-1 IFN at 1–2,000 units/ml did not significantly alter the initial growth rate of these cells. Similarly, exposure to TPA, PDD, PDB, mezerein, phorbol, 4α-PDD, or 4-*O*-MeTPA at 0.01–100 ng/ml also did not alter the growth rate (Fig. 1*A*). IFLrA (100–5,000 units/ml) did result in an earlier plateau in cell growth, due to earlier fusion of myoblasts (Fig. 1*B*).

Under the conditions used in the present study the cells obtained from normal adult human muscle fuse to form myotubes as the culture reaches confluency. In control cultures this process begins approximately 5–7 days after cell plating and is

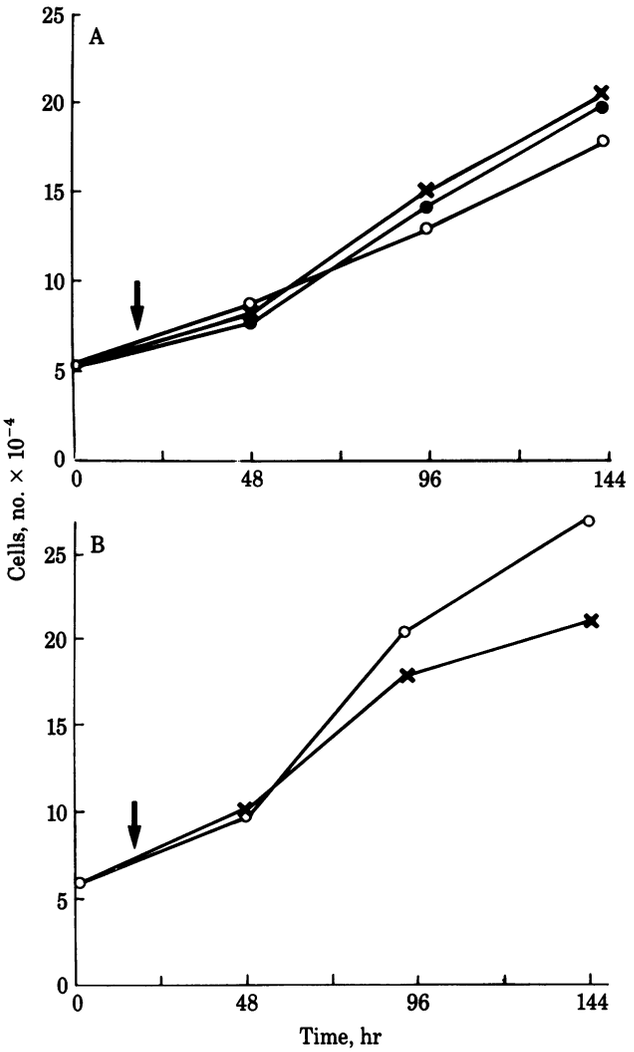

FIG. 1. (*A*) Growth curves of control human myoblast cultures prior to cell fusion (○) and of parallel cultures exposed to TPA at 10 ng/ml (●) or 100 ng/ml (×). The individual points represent means for three or four replicate cultures; the arrow indicates the time of drug addition. (*B*) Growth curves of control cultures (○) and of cultures treated with IFLrA at 1,000 units/ml (×). The variation between replicate samples differed by <10%.

complete within about 48 hr, at which time about 60–70% of the cells have fused. The morphology of myoblasts prior to the onset of fusion and of the multinucleated myotubes resulting from myoblast fusion are shown in Fig. 2 *a* and *b*. To quantitate the effects of IFN and TPA on myoblast fusion, parallel control and treated cultures were scored for the percentage of nuclei in the culture that were present in multinucleated myotubes at 24, 48, and 72 hr after the onset of myoblast fusion was detected in the control cultures. At 24 hr, IFLrA at 500–1,000 units/ml caused about a 2-fold increase in myoblast fusion (Table 1). Because the control cultures continued to undergo extensive myoblast fusion during the subsequent 48–72 hr, during the latter time interval they essentially caught up with the IFN-treated cultures. The ability of IFN to accelerate the onset of myoblast fusion was completely eliminated when it first was heated for 30 min at 60°C or treated with trypsin (5 mg/ml) for 30 min followed by soybean trypsin inhibitor (5 mg/ml).

In contrast to the results obtained with IFN, when the cultures were grown in the presence of TPA at either 10 or 100 ng/ml there was about a 2-fold inhibition of myoblast fusion (fusion index at 48 hr, 31% or 25%, respectively) (Fig. 2). Sim-

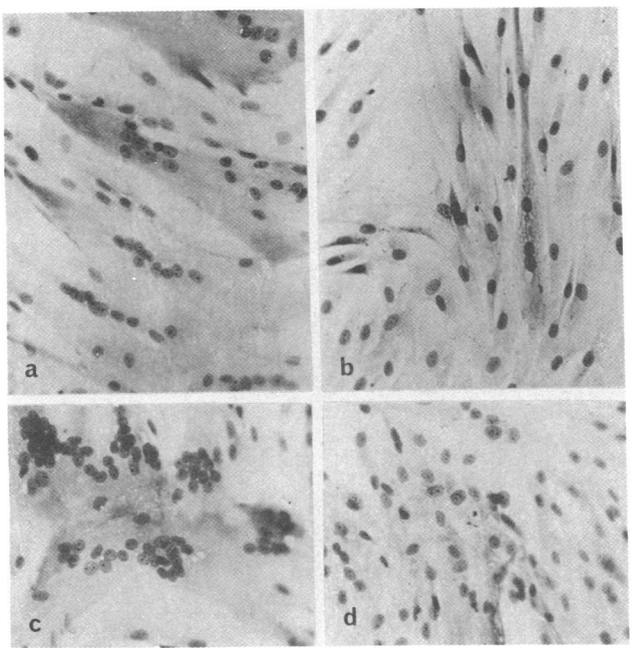

FIG. 2. TPA-induced inhibition of normal myoblast fusion and Ca^{2+}-induced fusion of cells grown in EGTA-containing medium. (Giemsa; ×130.) (*a*) Control myotube culture, 8 days after plating. (*b*) TPA-treated culture (100 ng/ml) 8 days after plating. (*c*) Control myoblast culture grown in the presence of EGTA and then re-fed with normal Ca^{2+} containing medium. (*d*) Myoblast culture grown in the presence of EGTA and re-fed with Ca^{2+}-containing medium plus TPA (100 ng/ml). *a* and *c* display cells that contain multiple nuclei characteristic of myotubes; *b* and *d* show predominantly myoblasts that contain single nuclei.

ilar concentrations of PDD and mezerein and higher concentrations of phorbol dibutyrate (200 ng/ml) produced a similar inhibition. On the other hand, at 100–200 ng/ml, phorbol, 4α-PDD, and 4-*O*-MeTPA, compounds that lack tumor-promoting activity (29), did not inhibit myoblast fusion (for phorbol at 100 ng/ml, fusion index at 48 hr, 71%). However, cultures treated with TPA, PDD, mezerein, or phorbol dibutyrate eventually partially escaped this inhibition of fusion so that by 72–96 hr the fusion index was 80–85% that of control cultures (31). Examples in which cells become refractory to other effects of TPA have been seen in other cell systems, although the mechanism is not known (for review, see ref. 32).

Spontaneous fusion of myoblasts can be prevented by Ca^{2+} depletion of the medium or chelation with EGTA (33). Addition of Ca^{2+} to the medium causes synchronous fusion within 8 hr (34). We found that TPA at 100 ng/ml causes almost complete inhibition of cell fusion in this type of fusion assay when added together with Ca^{2+}, compared with untreated controls (Fig. 2 *c* and *d*). In this case the cells also escaped from the effects of TPA and underwent some fusion about 72 hr later, so that the

Table 1. Effects of IFN on myoblast fusion

Time, hr	Fusion index, %				
		IFN			Heat-inactivated IFN 1,000 units/ml
	Control	At 10 units/ml	At 500 units/ml	At 1,000 units/ml	
24	36	35	67	59	38
48	69	71	79	78	ND
72	67	ND	ND	75	64

IFLrA, or a heat-inactivated preparation of IFLrA (100°C for 20 min), was added 16–18 hr after cell plating. The fusion index was then measured 24, 48, or 72 hr after the control culture first began to display myotube formation. The fusion indices (number of nuclei present in cells containing three or more nuclei divided by the total number of nuclei counted) are times 100. The values are the means of triplicate samples which varied by less than 10%. Qualitatively similar results were obtained with myoblast cultures from four different patients. ND, not determined.

eventual fusion index was 28–45% of that of the control culture. The effect of TPA in this system suggests that it can have a direct inhibitory effect in the process of fusion of human myoblasts. A similar effect has been seen in cultures of chicken embryo myoblasts (35). These results are consistent with the evidence that TPA can exert a number of other direct effects on membrane structure and function (for review, see ref. 32).

Effects of IFN and Tumor Promoters on CK Isoenzyme Transition. Prefused human myoblast cultures had a mean (± SD) total CK activity of 0.091 ± 0.012 unit/mg of protein. Twenty-four hours after initiation of fusion, the CK activity of control cultures had risen to 0.193 ± 0.017 unit/mg of protein. In parallel IFLrA-treated cultures, the value was 0.264 ± 0.013 unit/mg; cultures treated with TPA at 100 ng/ml had only slightly higher activities than controls, 0.126 ± 0.090 unit/mg. When myoblasts fuse to form multinucleated myotubes they undergo a transition in CK isoenzymes from the immature CK-BB to the more mature CK-MM (21, 31). Under our experimental conditions, in the control cultures CK-MM is barely detectable at 24 hr after fusion but by 48–72 hr there is a marked increase in CK-MB and CK-MM isoenzymes (Fig. 3) (21, 31). IFLrA (100–1,000 units/ml) caused an acceleration in the appearance of isoenzymes containing M subunit (MB and MM). A similar effect was seen with crude KG-1 IFN at 100 or 1,000 units/ml. By 48 hr after treatment, cell fusion and isoenzyme patterns of the control and IFN-treated cultures were similar. In contrast, TPA at 10 or 100 ng/ml delayed the appearance of the MB and MM isoenzyme forms in these cultures (Fig. 3*B*). By 96 hr, however, the TPA-treated cells had escaped with respect to this parameter so that the isoenzyme profiles of the TPA-treated cells were similar to those of the control cultures. An inhibition in appearance of the MB and MM forms was also seen with PDD or mezerein at 10–100 ng/ml and with phorbol dibutyrate at 100–200 ng/ml but not with phorbol, 4α-PDD, or 4-*O*-MeTPA

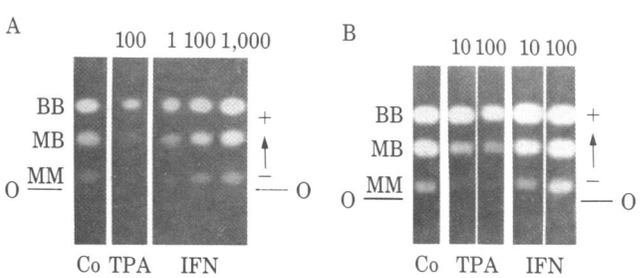

FIG. 3. Effects of TPA and IFLrA (IFN) on CK isoenzyme transition in human myoblast cultures were examined 24 hr (*A*) or 48 hr (*B*) after the onset of fusion of control cultures (Co). Concentration is indicated in ng/ml for TPA and in units/ml for IFN.

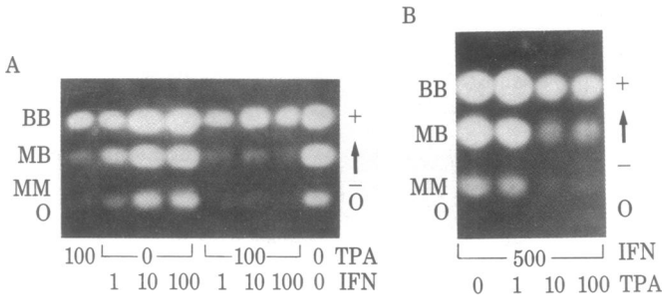

FIG. 4. Effects of TPA and IFLrA (IFN) alone and in combination on CK isoenzyme transition in human myoblast cultures. Isoenzyme profiles were examined 48 hr after the onset of fusion of control cultures. (*A*) TPA concentration was held constant at 100 ng/ml and IFN was varied between 1 and 100 units/ml. (*B*) IFN was held constant at 500 units/ml and TPA was varied between 1 and 100 ng/ml.

at 200 ng/ml. Thus, the effects of IFN and of TPA and related compounds on the transition of this isoenzyme system parallel in several respects the effects of these compounds on myoblast fusion.

In view of the opposite effects obtained with IFN and TPA, it was of interest to test the effects of these agents when applied simultaneously to 24-hr myoblast cultures. When TPA (100 ng/ml) was added together with IFLrA (1–100 units/ml), the tumor promoter completely blocked the enhanced expression of the MB and MM isoenzyme forms seen with IFN alone (Fig. 4*A*). When the concentration of IFLrA was kept constant (500 units/ml) and the concentration of TPA was varied from 1 to 100 ng/ml, only the 10 and 100 ng/ml doses of TPA inhibited the inducing effect of IFN (Fig. 4*B*). Similar results were obtained when TPA was combined with crude KG-1 IFN; however, this IFN preparation showed greater batch-to-batch variation in terms of its effects on myogenesis and at high concentrations (>1,000 units/ml) it caused some growth inhibition and vacuolization of the cells. We also found that TPA at 10–100 ng/ml also inhibited the early onset of myoblast fusion induced by KG-1 IFN (data not shown).

DISCUSSION

In the present study we investigated the effect of a bacterially produced human leukocyte IFN (IFLrA) on differentiation of normal adult human myoblasts by utilizing, as markers, the fusion of myoblasts to form myotubes and the appearance of the CK isoenzyme forms CK-MB and CK-MM (21, 31). We also evaluated the effects of the tumor-promoter TPA and related compounds on this system because these agents are known to be potent modulators of various programs of differentiation in diverse cell systems (13–20). We found that, in this system, nontoxic concentrations of IFN accelerate differentiation whereas the tumor promoters inhibit it. The opposing effects of IFN and TPA were also apparent when these agents were applied simultaneously; in this case, TPA inhibited the effects of IFN. The specificity of this IFN effect is indicated by the following findings: (*i*) it occurred in the same concentration range in which IFLrA exerts its antiviral effects (30), (*ii*) it occurred without evidence of cytotoxicity, (*iii*) it was not seen with heat- or trypsin-inactivated IFLrA, and (*iv*) similar effects were seen with a crude preparation of human leukocyte KG-1 IFN but not with a preparation of mouse L-cell IFN (unpublished data). The inhibitory effects seen with TPA and related compounds were also specific because they occurred in the range of 1–10 nm and were not seen with phorbol, 4α-PDD, or 4-*O*-MeTPA, analogs that lack tumor-promoting activity on mouse skin (33). Recent studies indicate that a partially purified preparation of avian IFN inhibits, rather than enhances, the differentiation of chicken myoblast cultures (36).

The mechanism by which IFN induces an antiviral state in responsive cells has been the focus of numerous investigations (1, 3). A number of complex biochemical responses are involved (reviewed in refs. 1–3). There is also increasing awareness of the fact that various IFNs can exert numerous other biologic effects, including antitumor activity (1, 2), and it seems likely that many of these effects are unrelated to the antiviral activity of IFN. In the past, mechanistic studies were hampered by the lack of highly purified preparations of IFN. The recent development of homogeneous preparations of IFN obtained by recombinant DNA technology greatly facilitates such studies (28, 29).

There is accumulating evidence that IFN can have specific effects on cellular differentiation. Previous studies have indicated that both crude and purified preparations of mouse IFN inhibit dimethyl sulfoxide-induced differentiation in Friend erythroleukemia cells (9, 10), inhibit spontaneous and melanocyte-stimulating hormone-induced differentiation in B-16 mouse melanoma cells (11), and also inhibit spontaneous adipocyte conversion of 3T3 cells (7, 8). In mouse myeloid leukemia cells, however, crude IFN has been found to exert an opposite effect because it enhanced the differentiation of these cells in the presence of known stimulators of differentiation—i.e., either lipopolysaccharide or polyinosinic acid (37). In recent studies, partially purified human leukocyte IFNs, IFN-α and IFN-β, did not induce differentiation of human promyelocytic leukemia (HL-60) cells when applied alone, but these preparations of IFN did enhance induction of differentiation stimulated by retinoic acid or TPA (12).

Thus, in all of the previously studied cell systems the direction of the effect on differentiation, either inhibition or stimulation, by IFN was the same as that obtained with TPA. However, in the present studies, IFN and TPA exerted opposing effects on the differentiation of normal human skeletal muscle cultures. In the literature there are a few other examples of opposing effects of TPA and IFN in cell culture systems (38). IFN inhibits TPA-induced DNA synthesis and TPA induction of ornithine decarboxylase synthesis in 3T3 cells (38). In addition, IFN inhibits the DNA stimulatory and mitogenic effects of epidermal growth factor (39), a polypeptide that shares a number of biologic effects with TPA (32). The latter findings suggest the possibility that the ability of IFN to inhibit the growth of tumors *in vivo* might be due in some cases to an ability to block the response of tumor cells to growth factors.

The mechanism(s) by which IFN and TPA modulate differentiation are not known. The primary site of action for both IFN (40–42) and TPA (43–45) appears to be cell membranes. Recent studies have demonstrated that cells contain high-affinity and saturable membrane-associated receptors for both classes of compounds (40–45). It seems likely that the reciprocal effects of these agents on differentiation in certain cell

systems reflect qualitatively different changes in membrane structure or function or both (46, 47). Indeed, recent studies have indicated that TPA and IFN can have opposing effects on membrane lipid fluidity when assessed by fluorescence polarization (42, 48–50). Opposing effects of IFN and TPA on the cell membrane might generate different transmembrane signals and thus either stimulate or inhibit differentiation. Further investigations of the effects of IFN, alone and in combination with other agents, on cellular differentiation may provide important insights into the mechanisms by which IFN exerts its antitumor effect and also lead to new strategies of combining IFN with other agents to enhance its antitumor effects.

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