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THE EFFECTS OF INTERFERON ON EPIDERMAL GROWTH FACTOR ACTION

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SUMMARY: Epidermal growth factor-stimulated thymidine incorporation in human fibroblasts is inhibited more than 80% by human interferon, whereas the stimulation of α-aminoisobutyrate uptake is unaffected. Maximum inhibition of thymidine incorporation is observed after treatment of cells with interferon prior to the onset of DNA synthesis. However, even after the initiation of DNA synthesis, interferon rapidly blocks any further increase in thymidine incorporation. Despite these effects, interferon treatment causes no alterations in epidermal growth factor binding, receptor down-regulation or receptor reappearance.

INTRODUCTION

It has been recognized for some time that interferon (IF)[4]-containing preparations can inhibit cell multiplication (1,2). Co-separation and co-purification of both the antiviral and antiproliferative activities from IF-containing preparations, as well as comparison of the antiproliferative activities of increasingly pure IF preparations, have led investigators to conclude that IF itself is the sole agent responsible for the growth-inhibition observed in earlier experiments (3). However, the mechanism of this IF-mediated growth inhibition is as yet unknown. In addition to a variety of effects on DNA synthesis and cell division (4-7), IF can cause changes in plasma membrane properties. For instance, increases in membrane glycosylation and membrane protein concentration (8), enhanced cell surface antigen expression (9,10) or reduced surface mobility

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[4]Abbreviations used: EGF, epidermal growth factor; IF, interferon; AIB, α-aminoisobutyric acid; [^{3}H]TdR, [methyl-^{3}H] thymidine; EMEM, Eagle's minimum essential medium; U, NIH reference units.

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of fluorescent Concanavalin A binding constituents (11) have been reported in IF-treated cells. Therefore, the possibility exists that IF could exert its growth inhibitory effect by altering mitogen-surface receptor interaction, mitogen receptor surface mobility (i.e. the patching, capping and internalization thought to be involved in mitogen action), or surface receptor reappearance (12-14). The present study was done to determine the effects of IF on mouse epidermal growth factor (EGF)-mediated mitogenesis in normal diploid human fibroblasts, and to evaluate the simultaneous effects of IF treatment of EGF receptor binding, receptor down-regulation and receptor reappearance.

MATERIALS AND METHODS

Explanted human foreskin fibroblasts were routinely cultured in Eagle's minimal essential medium (EMEM: Grand Island Biological Co., Grand Island, N.Y.) supplemented with 5% fetal calf serum (Reheis Chemical Co., Kankakee, Il.). Upon reaching confluency, cells were detached from the surface using 0.25% trypsin containing 0.01% (w/v) EDTA, diluted 1:5 with EMEM + 10% fetal calf serum (seeding medium), and transferred to new 75 cm^2 flasks (Oxnard, CA.). Cells were subcultured for study in multidish trays.

[^{125}I]EGF binding, [^{3}H]TdR incorporation and α-aminoisobutyrate (AIB) uptake were determined as previously described (15). "Down-regulation" of receptors in confluent monolayers (1.5 cm diameter) was achieved by the addition of EGF (final concentration 20 nM) directly to the culture medium. Recovery from down-regulation was accomplished by aspirating the EGF-containing medium from down-regulated cultures, rinsing twice in 1 ml growth medium for 15 min. at 37°C, and then incubating cells further in 1 ml growth medium for 20 hr.

Purified human fibroblast IF (specific activity 2.5 x 10^6 to 2.0 x 10^7 U/mg protein) was provided by Dr. W.A. Carter (Hahnemann Medical College and Hospital, Philadelphia, PA.) or was purchased from HEM Research Laboratories (Rockville, MD). EGF was prepared by the method of Savage and Cohen (16) or was purchased from Collaborative Research (Waltham, MA.). All IF and EGF preparations yielded similar results, regardless of the source.

RESULTS

Effects of IF on EGF-enhanced [^{3}H]-TdR incorporation and AIB uptake: Growth curves of human fibroblasts in growth medium containing various concentrations of IF were examined. Increasing growth inhibition with increasing IF concentrations above 250 U/ml was observed, with no additional inhibition of growth occurring at IF concentrations exceeding 1000 U/ml (not shown). Therefore, an IF concentration of 1000 U/ml was used in all experiments described.

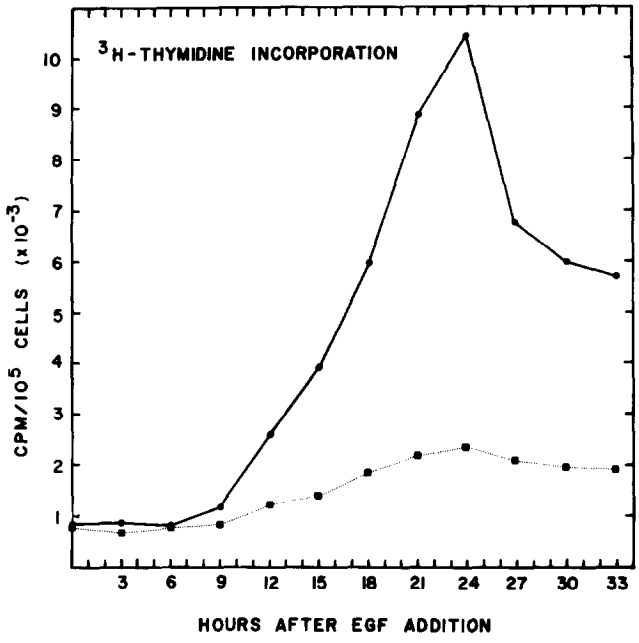

Fig. 1 - Inhibition of EGF-stimulated thymidine incorporation by Interferon. Confluent human fibroblast monolayers were stimulated by the addition of EGF (20 nM). At three hour intervals after the addition of EGF, replicate IF-pretreated (-■-■-) or untreated (-●-●-) cultures were incubated for 1 h at 37° with ^{3}H-labeled TdR (1 µCi/ml; 6 Ci/mmole) and the incorporation of radioactivity was determined as described (15).

The ability of EGF to stimulate DNA synthesis in confluent monolayers (cell densities exceeding 5 x 10^4 cells/cm^2) was examined both without and with 24h pretreatment with IF (1000 U/ml). In one-hour pulse experiments with [^{3}H]TdR, initiated at 3h intervals over the 33-hour period subsequent to EGF addition, IF-treated cultures displayed a marked inhibition of thymidine incorporation (Figure 1). Nonetheless, in both IF-treated and untreated cultures TdR incorporation was maximal 24 hours after EGF addition (Figure 1). Thus, there was no delay in the induction kinetics of the reduced amount of DNA synthesis observed in the presence of IF. The reduction in EGF-stimulated TdR incorporation at 24h (greater than 80%) was the same for cultures pretreated with IF for 24, 48 and 72h prior to EGF addition (data not shown). When added at 12, 15, and 21 hours after EGF stimulation (i.e. during the rise of DNA synthesis), IF rapidly abrogated any further increase in the rate of DNA synthesis without affecting the

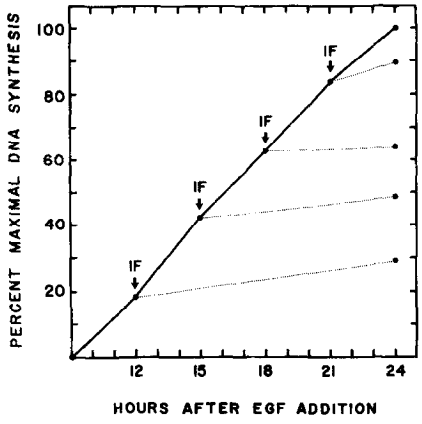

Fig.2 - Inhibition of EGF-stimulated thymidine incorporation after the timed addition of Interferon. At 12, 15, 18 and 21 hours after the addition of EGF (20 nM) to confluent monolayers, cultures were either treated by the addition of IF (1000 U/ml) or were incubated for 1 h with ^{3}H-labeled TdR to measure DNA synthesis (-●-●-). At 24 h, ^{3}H-TdR incorporation was similarly measured for IF-treated (...●...●...) or EGF-stimulated (-●-) cultures. DNA synthesis is expressed as the percent ^{3}H-TdR incorporation relative to the maximum amount incorporated at 24 h in untreated, EGF-stimulated cultures. Calculations were corrected for the baseline ^{3}H-TdR incorporation measured in the absence of EGF.

rate achieved prior to IF addition (Figure 2); an effect within about 1h after IF addition can be estimated.

In contrast to the effects on thymidine incorporation, a 24-hour pretreatment with IF had no effect on the ability of EGF to enhance AIB uptake (Table I) and only slightly decreased EGF-stimulated uridine incorporation (90% of non-IF-treated cells). Similarly, thymidine transport was unaffected by IF (not shown).

EGF receptor binding, down-regulation and recovery: Under similar conditions where substantial IF-mediated inhibition of EGF-stimulated [^{3}H]TdR incorporation was observed, the binding of EGF to untreated or IF-pretreated cultures was the same (data not shown). Receptor behaviour in terms of the ability of EGF receptors to down-regulate and to recover from down-regulation in the presence and absence of IF was also examined. Monolayers, induced to down-regulate by the addition of EGF either directly to the IF-containing medium or to pretreated cultures, were examined for [^{125}I]EGF binding after a 4-hour incubation at 37°C. IF-free, similarly down-regulated

TABLE I. EGF-stimulated AIB uptake

Addition	Time (min)	Stimulated AIB Uptake (ΔCPM/10^5 cells)
None	5	0
IF	5	0
EGF	5	9,800 ± 4,000
EGF + IF	5	10,500 ± 2,000
None	10	0
IF	10	0
EGF	10	31,800 ± 4,000
EGF + IF	10	32,700 ± 6,000

Monolayers (approx 3 x 10^5 cells/well) were pretreated for 24 h at 37° with IF (1000 U/ml) and were then preincubated with EGF (7.4 nM) for 60 min at 37° in an amino acid-free medium containing 0.1% w/v bovine albumin. ^{3}H-labeled AIB (37 μCi/ml; 10 μC: /mmole) was then added and the cellular uptake of radioactivity was determined (15) after incubation for 5 and 10 min at 37°. Stimulated AIB uptake (ΔCPM) was calculated by subtracting from the uptake in the presence of EGF and/or IF the AIB uptake in the absence of added stimulant (56,900 CPM/10^5 cells at 5 min and 92,700 CPM/10^5 cells at 10 min.). Values represent the average ± 1/2 range for measurements on duplicate monolayers.

control cultures were used for comparison. To allow for receptor recovery, identical IF-pretreated and untreated EGF-down-regulated monolayers were incubated an additional 20 hours, after being rinsed with growth medium, prior to measurement of [^{125}I]EGF binding. IF-pretreated monolayers were refed with growth medium containing IF. The results indicate that the pretreatment and presence of IF does not influence either EGF receptor down-regulation or receptor recovery in confluent fibroblast monolayers (Table II).

DISCUSSION

The main finding of this study is that pretreatment of human fibroblasts with IF abolishes the mitogenic effect of EGF without affecting either the receptor binding of EGF or the down-regulation and reappearance of the EGF receptor, and without blocking early events (increased amino acid transport) in the course of EGF-stimulated thymidine incorporation. The lack of an IF effect on ligand binding, down-regulation and receptor

TABLE II. Down-Regulation and Regeneration of EGF Binding

Treatment	EGF binding (CPM) at t = 0h	4h	20h
None	27,800± 700	2,960± 480	21,810± 2,170
IF	27,800± 700	2,880± 280	19,530± 680

Either IF-treated (1000 U/ml for 48 h at 37°) or untreated cell monolayers (approx 10^5 cells/monolayer) were incubated either without (0 h control) or with (4 h and 20 h samples) 20 nM unlabeled EGF. At 4 h cells were refed with EGF-free growth medium either with or without IF. The specific binding (CPM per monolayer) of ^{125}I-labeled EGF (5.1 ng/ml; 290 CPM/pg) was then measured for down-regulated monolayers (4 h) for non-down-regulated monolayers (0 h control) or for monolayers that were allowed to regenerate for 20 h at 37° in serum-containing growth medium (20 h). Values, representing the mean ± 1/2 range of measurements on quadruplicate monolayers are indicative of three independent experiments.

reappearance (presumably requiring de novo receptor synthesis (13,14) can be contrasted with the effects of IF on lymphocytes. Matsuyama (11) observed that IF treatment of lymphocytes inhibited patch and cap formation of Concanavalin A binding sites, so as to implicate reduced mitogen receptor mobility as a factor in the IF-mediated inhibition of lymphocyte blastogenesis; similar effects of IF on EGF receptor mobility, if present, cannot explain the inhibition of EGF-stimulated fibroblast DNA synthesis.

The action of IF appears to be rapid (within about 1 h) and independent of the time at which IF is added subsequent to the EGF stimulus. Thus, the site(s) at which interferon exerts its growth-inhibitory action can be placed distal to the mitogen-related membrane-localized events involved in the initiation of DNA synthesis. The rapidity of IF action suggests that de novo gene expression may not be required, as indicated by kinetic studies on the appearance of IF-induced enzymes in HeLa cells (17). It will be of particular interest to assess the effect of IF on the stimulation by EGF of a cytoplasmic protein factor that activates DNA synthesis in isolated frog nuclei (18). In addition, it will be important to determine if IF blocks other non-mitogenic actions of EGF, such as the effects on hormone production (increased prolactin; reduced growth hormone synthesis in cultured rat pituitary cells (19). Because of the potential use of IF as a

cancer chemotherapeutic agent it will be important in future work to establish the precise site(s) at which IF modulates the action of polypeptide mitogens like EGF.

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