Inhibition of mitogen stimulated growth of human colon cancer cells by interferon

A.W. Hamburger, M.E. Condon & K. O'Donnell

University of Maryland Cancer Center, 655 W. Baltimore Street, Baltimore, MD 21201, USA.

Summary Recombinant human interferon alpha inhibits growth of a human colon cancer cell line, Colo 205. To explore the mechanisms of IFN induced growth inhibition, quiescent Colo 205 cells were stimulated to proliferate in serum-free media by defined growth factors. Addition of insulin, transferrin and selenium (ITS) stimulated DNA synthesis, as assessed by $^{3}H$-thymidine incorporation, in a dose-dependent manner. IFN-α (at concentrations $>100$ U ml$^{-1}$) inhibited ITS stimulated DNA synthesis by 63%. Inhibition of cell cycle traverse was confirmed by flow cytometric analysis. Although IFN inhibited growth of ITS-treated cells, steady state levels of c-myc mRNA remained above levels observed in unstimulated cells. IFN inhibited DNA synthesis only when added prior to mitogen stimulation. IFN, added 6 h after exposure of quiescent cells to ITS, failed to inhibit cell growth. Addition of increasing concentrations of ITS failed to overcome the IFN-induced growth inhibition. These results suggest IFN may inhibit cell growth in part by antagonizing the action of growth factors.

In addition to their antiviral activity, IFNs inhibit growth of both normal and transformed cells. However, the mechanisms of IFN mediated growth inhibition are not fully understood (Clemens & McMurlan, 1985). One hypothesis to explain the antiproliferative activity of IFNs suggests that they act, in part, as mitogen antagonists. IFN, added simultaneously with mitogens, inhibits stimulation of DNA synthesis and cell division. For example, the administration of IFN concomitant with serum blocks passage out of $G_0$/$G_1$ of BALB/c 3T3 fibroblasts (Lin et al., 1986). When quiescent 3T3 cells are stimulated to initiate DNA synthesis by epidermal growth factor (EGF) and insulin, IFN potently inhibits DNA synthesis (Taylor-Papadimitriou et al., 1981, 1985a). EGF-stimulated thymidine incorporation by human fibroblasts is also inhibited more than 80% by human IFN (Lin et al., 1980). Maximum inhibition of thymidine incorporation is observed when cells are treated with IFN prior to onset of DNA synthesis. Tominaga and Lengyel (1984) and Oleask and Inglot (1980) similarly observed that treatment of quiescent BALB/c 3T3 cells with IFN inhibits cell replication induced by platelet-derived growth factor (PDGF). In a somewhat analogous system, Heyns et al. (1985) showed recombinant IFN inhibits smooth muscle cell growth induced by serum or platelet-poor plasma and PDGF. B-cell growth factor induced proliferation of hairy cell leukaemia cells is also inhibited by IFN (Paganelli et al., 1986). These studies suggest IFNs may control cell growth by acting as mitogen antagonists.

Some IFNs may be classified as naturally produced growth inhibitors. Hematopoietic cells induced to differentiate produce IFNβ which slows their own growth (Resnitsky et al., 1986). PDGF-stimulated 3T3 fibroblasts produce IFN 18 h after c-myc activation as part of a natural process of feedback inhibition (Zullo et al., 1986). Tumour necrosis factor (TNF) (mitogenic for human diploid fibroblasts) induces cellular synthesis of β-IFN mRNA (Kohase et al., 1986). Anti-β-IFN antibody enhances the mitogenic effect of TNF on confluent serum-starved fibroblasts. All of the above observations are consistent with the assumption that the induction of IFN by growth factors is a physiological negative feedback mechanism involved in control of cell proliferation.

IFN inhibits the in vitro growth of both malignant cell lines and cells derived from patient biopsies (Clemens & McMurlan, 1985). IFN decreases clonal growth of human colon tumour cells, isolated directly from patients, in soft agar (Scheitauer et al., 1985). Other workers have found the growth of several human colon cancer cell lines is inhibited by both naturally produced and recombinant IFNs (Denz et al., 1985). The Colo 205 cell line, derived from a patient with adenocarcinoma of the colon, is sensitive to the antiproliferative effect of IFNα (Brouty-Boyé et al., 1985). IFN profoundly affects both proliferation and tumorigenic capacity of this cell line.

We reasoned that part of the cytostatic effect of IFN on Colo 205 cells was due to its ability to interfere with the proliferative stimulus of serum growth factors. The present study demonstrates that the growth of Colo 205 cells is dependent on insulin and transferrin. We also report that recombinant IFNα inhibits proliferation of Colo 205 colon carcinoma cells and abolishes the mitogenic effect of insulin and transferrin.

Materials and methods

Reagents

Human recombinant IFNα-2 (1.7 × 10⁷ units mg⁻¹ protein) was a gift of Schering Corp. (Kenilworth, NJ). Insulin, transferrin and selenium were obtained from either Sigma (St Louis, MO) or Collaborative Research (Sudbury, MA).

Cell cultures

Colo 205 cells (CCL 222) were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium containing 10% (v/v) foetal bovine serum (FBS). The cells were used within 20 passages of the original frozen stock.

Growth inhibition of Colo 205 cells

Colo 205 cells were seeded at 1 × 10⁴ cells ml⁻¹ in 5 ml complete media in 25 cm² tissue culture flasks in the presence or absence of the indicated concentrations of IFN. The cell number was determined on the indicated days by releasing the cells with trypsin and counting them in a haemocytometer. Cell viability was assessed by exclusion of trypan blue dye.

Thymidine incorporation assays

Colo 205 cells were plated into 24 well tissue culture dishes at 1 × 10⁴ cells ml⁻¹ in 1 ml RPMI 1640 media with 10% FBS. Cells became quiescent after 3 days as determined by FACScan analysis (Table 1). The monolayers were washed twice with RPMI 1640 salts and changed to RPMI 1640 media containing 0.5% FBS in the presence or absence of IFN at

Correspondence: A.W. Hamburger.
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the indicated concentrations. After incubation at 37°C for 48 h, insulin (5 μg ml⁻¹), transferrin (5 μg ml⁻¹) and selenium (5 ng ml⁻¹) (ITS) were added. Sixteen hours later, the monolayers were washed twice with 0.2% SDS,一个小时 with 0.5% SDSPAGE sample buffer (Sigma), and resuspended and protected from light, until flow cytometric analysis. Both the fluorescence and narrow angle light scatter were simultaneously measured using a multiparameter fluorescence activated cell sorter (FACS IV Becton-Dickinson). Incident light at 488 nm was provided by an argon ion laser (Model 164-5 Spectra Physics) operated at 0.3 W in the light stabilized mode.

Detection of c-myc mRNA

Colo 205 cells, cultured in RPMI 1640 and 10% FBS, were seeded into T75 flasks at 1 × 10⁵ cells ml⁻¹. After incubation at 37°C for 3 days, the confluent monolayers were washed twice with RPMI 1640 without serum and further incubated with RPMI 1640 and 0.5% FBS or with or without 1,000 U ml⁻¹ of IFNα at 37°C. After 48 h, half the cultures were supplemented with ITS and 2 h later, the cells were harvested and total cytoplasmic RNA was isolated by the guanidium thiocyanate method (Chirgwin et al., 1980). The RNA samples were dot blotted at different concentrations onto nitrocellulose filters. The filters were prehydrated for 48 h under agitation at 42°C in 5×SSC—50 mM sodium phosphate containing 50% deionized formamide, 0.2% SDS, 0.5 mM EDTA, 5×Denhardt’s and denatured salmon sperm DNA (100 μg ml⁻¹). The blots were hybridized to a cDNA probe nick translated to 5×10⁶cpm μg⁻¹ DNA with α²⁵P-dCTP (New England Nuclear). A 1.5 kb ClaI-Eco R1 DNA fragment encoding the third exon of the human c-myc gene was used. (Della-Faveri et al., 1985). Filters were hybridized 16 h at 40°C and washed at 65°C in 2×SSC, 0.1% SDS. The filters were autoradiographed on X-ray film at −70°C using intensifying screens. Autoradiograms were quantitated by densitometric scanning using a Helena densitometer.

Statistical analysis

The two tailed Student's t test was used on paired samples to compare control to experimental groups. Data are expressed as mean ± s.d. Statistical significance was established at the 5% level.

Table 1 IFN-induced inhibition of cell cycle transit

| Addition | G₀ | S | G₂/M |
|----------|----|---|------|
| None     | 88 | 10 | 2    |
| IFN      | 85 | 12 | 3    |
| ITS      | 53 | 42 | 5    |
| ITS and IFN | 78 | 19 | 2    |

Quiescent Colo 205 cells were serum starved, and incubated in the presence or absence of IFN for 48 h as described. ITS was added and the number of cells in various phases of the cell cycle determined 16 h later by flow cytometric analysis of propidium iodide labelled nuclei.

Results

Antiproliferative effects of IFNα

We initially determined the effect of recombinant IFNα₂ on proliferation of Colo 205 cells in monolayer culture. Cells were seeded in the presence or absence of IFN as described and the total number of viable cells determined daily. Results indicated IFNα₂ decreased cell growth to 17% of control values (Figure 1). This was due to a cytostatic, rather than cytotoxic, effect as cell viability, judged by trypan blue exclusion, was unchanged (data not shown).

Increasing concentrations of IFN decreased cell growth in a dose-dependent manner. A 50% decrease in cell growth was observed at 125 U ml⁻¹ of IFN (Figure 2).

Effect of IFNα₂ on ITS induced DNA synthesis

We reasoned part of the cytostatic effect of IFN was due to its ability to interfere with the proliferative stimulus of serum factors. The mitogenic activity of insulin on Colo 205 cells and its modulation by IFN were examined. A series of preliminary experiments established the conditions for testing the effect of human IFN on replication of quiescent Colo cells induced to divide by exposure to ITS. On the basis of these experiments, we chose to expose confluent cells to 0.5% serum and increasing concentrations of IFN for 48 h. Growth was then stimulated with ITS for 16 h. Incubation of confluent serum-starved cells with ITS for 16 h stimulated thymidine incorporation 3.5 fold. The data in Figure 3 reveal that pretreatment of cells with IFN at concentrations...
of 100 to 5,000 U ml\(^{-1}\) blocked much of the stimulation of DNA synthesis induced by ITS. Incubation of unstimulated cells with IFN also decreased thymidine incorporation. However, this decrease was small (10%) in comparison to the decrease induced in the presence of ITS. Similar results were obtained in initial experiments when cells were assayed 48 h after ITS stimulation suggesting increases in thymidine incorporation were inhibited, rather than delayed, by IFN (data not shown). Decreases in thymidine incorporation were not due to decreases in cell number per well as equal numbers of cells were present at the time of ITS stimulation (1.6 ± 0.3 \times 10^5 for controls vs. 1.4 ± 0.5 \times 10^5 for IFN treated cultures).

Flow cytometric analysis

To ascertain if results obtained by thymidine incorporation methods accurately reflected DNA synthesis, we also estimated the movement of cells through the cell cycle by flow cytometry. The incorporation of thymidine into acid insoluble material has been widely used as a convenient method of assessing the growth inhibitory effect of IFN. However, this technique not only assesses effects on DNA synthesis, but also may reflect alterations in thymidine transport across the plasma membrane, phosphorylation of nucleosides by thymidine kinase, and changes in intracellular pools (Taylor-Papadimitriou et al., 1985b). Therefore, cells were grown to confluence and serum starved for 48 h in the presence or absence of IFN (1,000 U ml\(^{-1}\)). ITS was added and cells harvested 16 h later. Cells were stained with propidium iodide and the numbers of cells in G\(_1\), S, or G\(_2\)/M were assessed as described. Table I shows IFN significantly reduced the number of cells in S phase 16 h after addition of ITS. Flow cytometry data confirmed the inhibition of cell growth observed with thymidine incorporation assays.

Effect of \(a\)IFN on the level of c-myc mRNA

Since IFN\(_{2}\) inhibited the stimulation of DNA synthesis induced by ITS, we tested whether IFN also impaired the increase of c-myc mRNA usually associated with cell replication. The data in Figure 4 reveal this was not the case. As expected, quiescent control cells expressed low levels of c-myc transcripts. Increased levels of c-myc mRNA were observed in cells treated with ITS only. This increased level of steady state c-myc mRNA was also observed in cells treated with both ITS and IFN, under conditions which inhibit cell replication. An increase in steady state c-myc RNA levels was also observed in IFN treated cells. Area integration of the densitometric scans of the autoradiogram revealed approximately equal levels of c-myc mRNA were found in ITS stimulated cells and cells receiving ITS and IFN (Table II).

**Table II**  Changes in c-myc mRNA expression after treatment with ITS and IFN

| Assay condition | Relative level of c-myc expression |
|-----------------|----------------------------------|
| Control         | 1                                |
| ITS             | 3.3                              |
| IFN             | 2.2                              |
| IFN + ITS       | 3.15                             |

Confluent Colo 205 cells were exposed to medium containing 0.5% serum (control). Interferon (IFN) (1,000 U ml\(^{-1}\)) were added to half the flasks for 48 h. Half the flasks in each of the two groups were then exposed to insulin, transferrin and selenium as described (ITS or ITS + IFN). Total RNA was extracted 2 h later. RNA (2 \(\mu\)g) was dot blotted onto nitrocellulose and hybridized to a \(^{32}\)P labelled c-myc probe. The resulting autoradiogram was quantitated by densitometry. Area integrals of these profiles were calculated relative to the level in control cells.

Effect of time of addition of IFN to quiescent Colo 205 cells

To determine optimal timing of the IFN treatment, cells were exposed to IFN either before or after addition of ITS at the times indicated (Figure 5). The growth inhibition observed was compared to that obtained by treating cells with IFN for 48 h prior to addition of ITS. IFN\(_{2}\), added simultaneously with ITS (Time 0), inhibited growth only 50% as well as IFN added for the entire 48 h pretreatment period. IFN added 6 h after addition of ITS failed to inhibit thymidine incorporation. We also varied the length of time of IFN pretreatment. Thymidine incorporation was not as effectively inhibited when cells were treated with IFN for only 6 h (Figure 5).

Effect of increasing concentrations of ITS on IFN mediated growth inhibition

To determine if increasing concentrations of ITS could overcome the IFN mediated inhibition of cell growth, quiescent Colo cells were stimulated to proliferate by adding increasing concentrations of ITS in the presence or absence of 1,000 units of IFN. The results in Figure 6 indicate that the degree of inhibition of thymidine incorporation was inversely related to the concentration of mitogens. The inhibitory effect of IFN could not be overcome by increasing the concentration of the mitogenic stimulus. Concentrations of ITS 10 times the maximal stimulatory concentration failed to overcome the IFN-induced inhibition of thymidine incorporation.
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Effect of time of addition of IFN on inhibition of DNA synthesis. Colo 205 cells were allowed to grow to confluence and IFN added at the indicated times either prior to exposure to ITS or at the times indicated after exposure to ITS. Thymidine incorporation was assessed 16h after. Results represent the percent of maximal inhibition observed when cells were exposed to IFN for 48h prior to addition of ITS. The maximal inhibition observed was 65 ± 5%. Values are the average of three independent experiments (4 wells/point).

Figure 5 Effect of time of addition of IFN on inhibition of DNA synthesis.

Thymidine incorporation (% control)

Effect of increasing concentrations of ITS on IFN induced inhibition of growth. Colo 205 cells were seeded at a density of 1 x 10⁵ cells/well and cultured as described in 24 well plates to achieve quiescence. Cells were then incubated in serum-free media for 48h in the presence or absence of IFN (1,000 IU/ml). Cells were stimulated with increasing concentrations of ITS and ³H thymidine incorporation in duplicate wells determined 16h later. Values are the averages of three independent experiments.

Figure 6 Effect of increasing concentrations of ITS on IFN induced inhibition of growth.

Discussion

We have found that pretreatment of quiescent human colon cancer cells with IFN abolishes the mitogenic effect of insulin and transferrin. Our results are in accord with earlier reports indicating the administration of IFN concomitant with serum (Lin et al., 1986), EGF and insulin (Lin et al., 1980; Taylor-Papadimitriou et al., 1981), or PDGF (Tomina et al. & Lengyel, 1984) blocks G1/G-S passage of human and murine fibroblasts. Similarly, growth factor stimulated proliferation of smooth muscle cells (Heyns et al., 1985), or leukaemic cells (Paganelli et al., 1986), is also inhibited by concomitant administration of IFN.

Although pretreatment with IFN diminished the mitogenic effect of ITS, the mechanism of this effect is unknown. The requirement for a long exposure to IFN for effective growth inhibition in our study suggests receptor interactions leading to the antagonistic effect. Similarly, Pfeffer et al. (1987) recently demonstrated IFN inhibited insulin-induced growth of Daudi cells. IFN pretreatment of cells reduced binding of insulin to low affinity receptors. Insulin binding was most effectively decreased by a 48h pre-exposure to IFN. Zoon et al. (1986) demonstrated IFN-z inhibited the EGF-stimulated growth of MDBC cells. IFN-z reduced binding of EGF to these cells by decreasing both receptor number and affinity. IFN may have similarly inhibited cell proliferation in our study by decreasing insulin binding.

Alternatively, a secondary interaction between IFN, its receptor, and cytoskeletal elements might have occurred. Cytoskeletal elements, particularly the microtubules, are thought to play a role in signal transduction. IFN can induce tubulin mRNA and interferon’s antiviral action can be inhibited by tubulin disrupting agents (Jaboin et al., 1985). It is therefore possible that continuous occupancy of the IFN receptor by exogenous ligand resulted in stabilization of the tubulin network, ultimately inhibiting DNA synthesis induced by growth factors. Taylor-Papadimitriou et al. (1985a) have found that tubulin disrupting agents such as colchicine or nocodazole are very effective at reversing the inhibitory effect of IFN on DNA synthesis.

As expected, exposure of quiescent cells to ITS increased c-myc mRNA levels. Although IFN prevented ITS-stimulated thymidine incorporation into DNA, IFN failed to decrease c-myc RNA levels down to those observed in unstimulated cells. Tomina and Lengyel (1984) similarly reported IFN pretreatment of 3T3 cells for 48h did not inhibit the ability of PDGF to increase levels of c-myc mRNA. Eismt et al. (1985) found that IFN inhibited growth of HL-60 cells, but failed to reduce steady state levels of c-myc mRNA. In contrast, IFN-z produced a decrease of c-myc mRNA levels and caused G1/G0 arrest of Daudi lymphoma cells. In addition we observed increased levels of c-myc mRNA in cells treated with IFN alone. Tomina and Lengyel (1984) found IFN pretreatment of 3T3 cells subsequently exposed to PDGF, resulted in higher levels of c-myc mRNA than in cells treated with PDGF only. In contrast to our study, IFN alone did not increase levels of c-myc mRNA. The mechanism of this IFN-mediated increase in c-myc mRNA levels is not known. Tomina and Lengyel (1984) suggested that the increase may reflect an IFN-mediated inhibition of labile repressor proteins that regulate steady state levels of c-myc mRNA. It is known that IFN differentially regulates protein synthesis. Thus, the increase in levels of c-myc mRNA in cells treated with IFN may be a consequence of an impairment of synthesis of these repressor proteins. Thus, IFN blocked thymidine incorporation induced by ITS, but did not prevent an increase in c-myc mRNA levels. These findings suggest expression of the c-myc oncogene may be a primary consequence of growth factor receptor interaction, rather than a cause or consequence of cell proliferation.

Our results indicated addition of IFN 6h after administration of insulin failed to inhibit cell growth. Lin et al. (1986) similarly demonstrated that addition of IFN 6h after serum stimulation of 3T3 fibroblasts failed to inhibit cell growth. In contrast, Lin et al. (1980) earlier found that IFN rapidly blocks increases in thymidine incorporation even after entry of human fibroblasts into S phase.

The inhibitory effect of IFN on Colo cultures could not be overcome by high concentrations of ITS. The addition of supramaximal concentrations of ITS (10 times the dose required to elicit maximal proliferation) failed to prevent the IFN-induced inhibition of cell growth. The inhibitory effect of IFN is probably not due to a direct competition with ITS. IFN may affect related but not identical cellular pathways as Foxnation of IFN.

In conclusion, we have demonstrated IFN inhibits growth of a colon cancer cell line, in part, by interfering with the ability of ITS to induce growth. Study of the IFN induced regulation of cellular response to insulin and transferrin will provide further insight into the mechanisms of IFN inhibition of cell growth.
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