GROWTH-INHIBITORY ACTIVITY OF INTERFERON-BETA AGAINST HUMAN COLORECTAL CARCINOMA CELL LINES

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Recombinant human interferon beta (rIFN-β) inhibited in a time- and dose-dependent manner the proliferation of 18/18 human colon carcinoma cell lines in monolayer culture and 8/9 lines in a soft agar assay but had no effect on 4 human fibroblast cell lines. Maximal inhibition of cell proliferation by rIFN-β required repetitive treatment (every 2 days) with lymphokine (50 units/ml). Furthermore, the inhibitory activity of rIFN-β was neutralized by polyclonal antibodies against natural IFN-β. In contrast to rIFN-β, rIFN-α was inactive against all colon cell lines tested, and rIFN-γ, with the exception of HT-29 cells, was similarly ineffective. These data demonstrate that rIFN-β is a potent growth inhibitor of colon carcinoma cells *in vitro,* and suggest that studies on its mechanism of action may lead to a better understanding of the regulation of colon tumor cell proliferation.

Colorectal carcinoma accounts for approximately 60,000 deaths per year in the USA and is refractory to standard chemotherapeutic intervention (Moertel, 1983). Several new approaches to therapy, including vaccination (Hoover *et al.,* 1985), monoclonal (Frödin *et al.,* 1986) and anti-idiotypic antibodies (Herlyn *et al.,* 1987) and immunoregulatory agents (Chachoua *et al.,* 1986), are currently under investigation. Our overall objective is to investigate the hormonal mechanisms that regulate the proliferation of colon carcinoma cells and identify novel sites for pharmacological intervention.

Cell proliferation is regulated by a balance of positive and negative growth factors and the cell's ability to respond to these signals. Hyperproduction of positive regulators, perhaps through autocrine mechanisms (Sporn and Todaro, 1980), or compromised availability of negative mediators may predispose to hyperplasia and eventually to frank malignancy. Evaluation of this concept in colorectal cancer has revealed that colon tumor cells synthesize and secrete increased levels of several positive growth regulators including transforming growth factor-alpha (TGFα), PDGF and IGF-I (Coffey *et al.,* 1987; Hanauske *et al.,* 1987; Anzano *et al.,* 1988; Tricoli *et al.,* 1986), but whether these mediators play a direct role in stimulating tumor growth is still unclear. Similarly, several lymphokines, principally IFN-γ (alone or in combination with rTNF-α), but also rTNF-β inhibit the *in vitro* proliferation of a select number of colon tumor cells (Ücer *et al.,* 1985; Pfizenmaier *et al.,* 1985; Fransen *et al.,* 1986; Williamson *et al.,* 1983; Schiller *et al.,* 1987*a*; Schmiegel *et al.,* 1987). Few investigations have been performed with rIFN-α (Ohashi *et al.,* 1987; Horoszewicz *et al.,* 1978; Chapekar *et al.,* 1983).

The goal of our study was to investigate in greater detail the anti-proliferative activity of different interferons in colorectal carcinoma and to this end we have examined the growth inhibitory properties of rIFN-α, rIFN-β and r-IFN-γ on an extensive panel of human colorectal carcinoma cell lines (Trainer *et al.,* 1988). The results show that rIFN-β blocks the proliferation of 18/18 colon cells lines in monolayer culture and 8/9 cell in soft agar. rIFN-α and rIFN-γ were much less effective. These data confirm and extend the anti-proliferative activity of IFN-β (Czarniecki *et al.,* 1984; Borden *et al.,* 1982) and suggest that studies on its mechanism of action may lead to new opportunities in the treatment of colorectal cancer.

MATERIAL AND METHODS

Reagents and cell lines

Human recombinant IFN-β (specific activity, 1.0×10^8 U/mg) was obtained from Triton Biosciences (Alameda, CA). Human recombinant IFN-α (IFN-α Con$_1$, specific activity, $0.5–3.0 \times 10^9$ U/mg) and human recombinant IFN-γ (specific activity, $1–5 \times 10^7$ U/mg) were purchased from Amgen (Thousand Oaks, CA). Interferon titers are expressed in units of antiviral activity as determined by the 50% inhibition of encephalomyocarditis virus cytopathology in human WISH cells (Dalton and Paucker, 1981). Titers are given in international reference units based on the NIH human fibroblast, leukocyte and gamma interferon reference standards G-023-902-527, G-023-901-527 and Gg23-901-530 respectively, distributed by the Resource Reagents Branch of the National Institutes of Allergy and Infectious Diseases (Bethesda, MD). Control antiserum and sheep antiserum to human fibroblast IFN-β were prepared as described by Dalton and Paucker (1981).

Neoplastic cell lines, except HCT-15 (rectal adenocarcinoma), were derived from human colon adenocarcinomas. CRL-1224, CRL-1222 and CCL-202 cells are adult human lung fibroblast lines and WI-38 are fibroblasts from human fetal lung. All cell lines, except LIM-1215 (Whitehead *et al.,* 1985), were obtained from the American Type Culture Collection (Rockville, MD). Cultures were maintained in DMEM containing 10% FBS (GIBCO, Grand Island, NY) at 37°C in a humidified 5% CO_2 atmosphere and were passaged once or twice a week. All cells were determined to be free of Mycoplasma and other adventitious agents (Trainer *et al.,* 1988). Cells were cultured in monolayer, except COLO-201, COLO-320DM and COLO-320HSR, which grew in suspension.

Proliferation assays

Two assays were used to examine the growth inhibitory properties of the different interferons. One assay measured the proliferative capacity of cells in monolayer culture, whereas the other determined colony-forming potential in soft agar. In the monolayer assay, 5,000 cells with or without interferon were plated in 24 well plates in 1 ml of DMEM containing 10% FBS. After 6–9 day incubation at 37°C, cells were fixed and stained with 0.5% crystal violet. Growth inhibition was determined by measuring the area of stained cells using an Omnicon

Image Analysis System (Bausch and Lomb, Rochester, NY), and values are expressed relative to untreated controls. The validity of this system was confirmed by performing direct cell counts using a model ZB Coulter Counter (Hialeah, FL) on parallel cultures.

Colony formation in soft agar was performed as described earlier (Anzano et al., 1982). Briefly, 6,000 cells in DMEM containing 10% FBS were plated in 0.5% agar (Difco, Detroit, MI) in 35-mm Petri dishes (Nunc, Roskilde, Denmark). Cultures were incubated with or without interferon for 8–10 days and the formed colonies stained overnight with 0.05% p-iodonitrotetrazolium violet (Sigma, St. Louis, MO) and quantitated using an Omnicon Image Analyzer. Growth inhibition was calculated relative to untreated controls. Assays were performed at least twice and in duplicate. The standard error was within ±10%.

RESULTS

Inhibition of colorectal carcinoma cell proliferation in monolayer culture was quantified either by image analysis of stained colonies or direct cell counting; both assays yielded similar results (Fig. 1). Under monolayer conditions, rIFN-β (50 U/ml) blocked the growth (20–92% inhibition) of 18/18 colon adenocarcinoma cell lines while in the soft agar assay, rIFN-β

A. Image Analysis

B. Cell Counts

FIGURE 1 – Anti-proliferative effect of rIFN-β quantitated by image analysis and direct cell counts. HCT-15 cells were challenged without (■) or with 50 U/ml (▨) or 100 U/ml (▧) rIFN-β for 9 days in monolayer culture. Growth inhibition was evaluated using area measurement in an Image Analysis System (top panel) or direct cell counts using a Coulter Counter (bottom panel) as described in "Material and Methods". Numbers at the top of each bar represent percent growth. Similar results were obtained with SW-620, DLD-1, HT-29 and SW-480 cell lines.

(100 U/ml) inhibited colony formation (40–90%) in 8/9 cell lines tested. The growth of 4 human lung fibroblast lines (Table I) and a normal rat kidney epithelial cell (data not shown) was unaffected. Inhibition was dose-dependent both in the monolayer and in the soft agar assays (Fig. 2). Similar antiproliferative effects were also displayed by partially purified natural human IFN-β (data not shown).

In the monolayer assay (Fig. 2a), inhibition of SW-480, HCT-15, DLD-1, HT-29 and SW-620 proliferation was dose-dependent over a rIFN-β concentration range of 10–50 U/ml. Higher doses (e.g., 100 U/ml) had little additional effect. Although all colon lines tested were responsive to the inhibitory effects of rIFN-β, a spectrum of sensitivity was observed (Fig. 2a and Table I). The most sensitive cell lines were DLD-1, SW-480, SW-620, HCT-15, LIM-1215 and SKCO-1, while the less responsive cultures included SW-948, SW-837, SW-1417 and SW-1463. Similar profiles were found in the soft agar assay. Figure 2b shows that the dose-response curves under these conditions were comparable to those observed in monolayer culture.

The suspension line COLO-320HSR was the only culture refractory to IFN-β's anti-proliferative effect (Table I), and these data were confirmed by direct cell counts (data not shown).

Growth inhibition was also time-dependent (Fig. 3). The antiproliferative effect of rIFN-β was readily detectable following a 3-day challenge of sensitive cells, but maximal inhibition required 6–9 days. Inhibition could be enhanced by repeated treatment of target cultures with rIFN-β. Cells challenged with rIFN-β (50 U/ml) every other day for 8 days displayed almost 100% inhibition, whereas cultures receiving the same dose but at 4- or 8-day intervals were growth-inhibited to a significantly lesser extent (Table II).
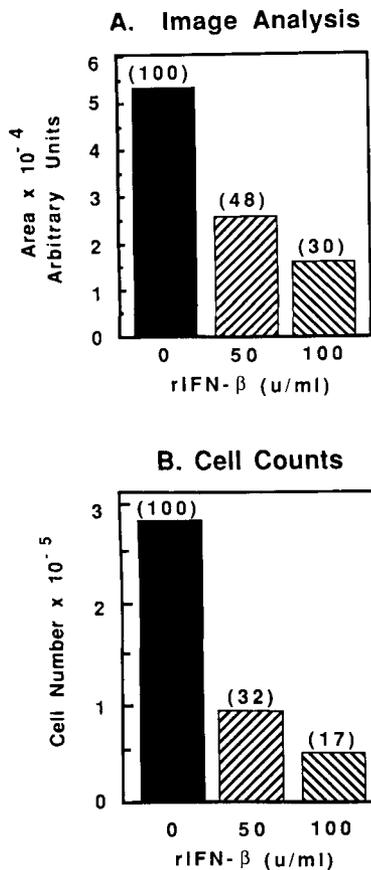
TABLE I – INHIBITION OF COLON CARCINOMA CELL PROLIFERATION BY rIFN-β[1]

Cell line	Growth inhibition (%)			
	Monolayer culture (50 U/ml)		Soft agar (100 U/ml)	
Colorectal carcinoma				
DLD-1	55–80[2]	(10)[3]	50–80	(2)
HT-29	30–60	(15)	50–75	(2)
HCT-15	40–86	(15)	40–60	(2)
SW-480	50–75	(10)	50–70	(3)
SW-620	40–70	(10)	70–90	(5)
SW-948	21–48	(5)	40–50	(2)
COLO-201	N.T.[4]		55–70	(2)
COLO-320DM	N.T.		50–60	(2)
COLO-320HSR	N.T.		<8	(2)
LIM-1215	70–78	(2)	N.T.	
LS-174T	62–70	(2)	N.T.	
LS-180	55–70	(2)	N.T.	
SKCO-1	90–92	(2)	N.T.	
SW-48	30–80	(3)	N.T.	
SW-742	60–65	(2)	N.T.	
SW-837	24–40	(2)	N.T.	
SW-1116	54–56	(2)	N.T.	
SW-1417	33–35	(2)	N.T.	
SW-1463	29–31	(2)	N.T.	
LOVO	64–65	(2)	N.T.	
WIDR	38–88	(4)	N.T.	
Lung fibroblasts				
CRL-1224	<10	(10)	N.T.	
CRL-1222	<22	(2)	N.T.	
CCL-202	<10	(2)	N.T.	
WI-38	< 5	(10)	N.T.	

[1]Cultures were challenged with r-IFN-β as described in "Material and Methods". –[2]Range.–[3]Number of assays.–[4]Not tested.
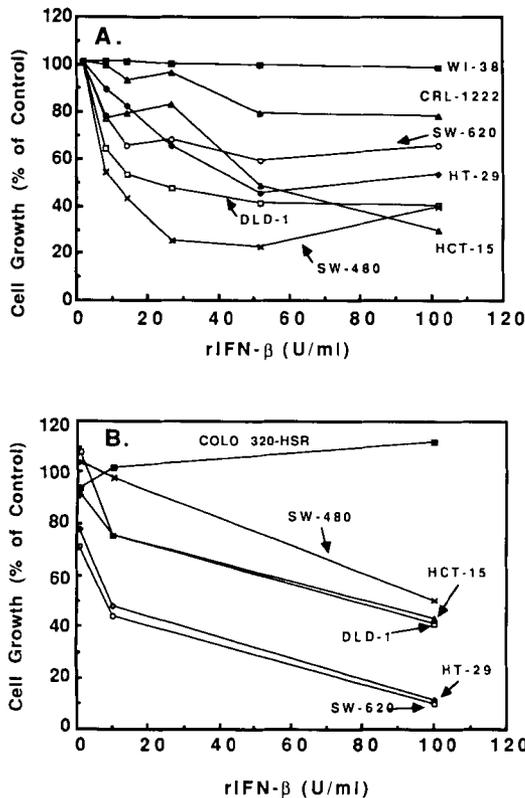
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FIGURE 2 – Dose-dependent anti-proliferative activity of rIFN-β on human colorectal carcinoma cells. Cultures were challenged with rIFN-β in either the (a) monolayer or (b) soft agar assay as described in "Material and Methods". Colorectal cell lines are SW-480, DLD-1, HT-29, HCT-15, SW-620 and COLO 320-HSR. CRL-1222 and WI-38 are non-neoplastic fibroblast lines. Values are representative of 3 or more experiments and each point is the average of duplicate plates.

Figure 4 shows that the growth-inhibitory activity of rIFN-β was completely blocked by antibody against natural IFN-β. Challenge of HCT-15, SW-620, DLD-1, HT-29 and SW-480 with rIFN-β (100 U/ml) caused a significant anti-proliferative
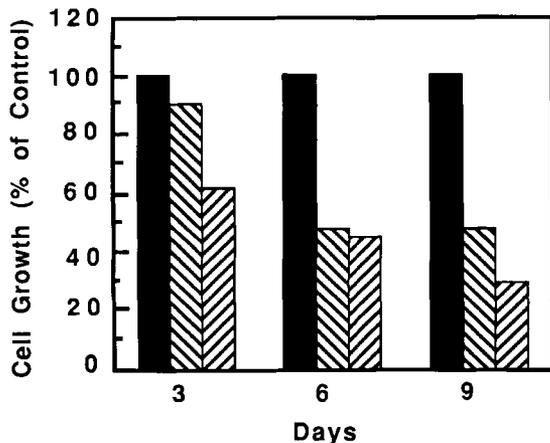

FIGURE 3 – Time-dependent inhibition of colon carcinoma cell proliferation by rIFN-β. Monolayer cultures of SW-620 cells were challenged with rIFN-β (■, 0 U/ml; ▨, 50 U/ml; ▧, 100 U/ml) for the indicated items. Growth inhibition was quantitated by image analysis as described in "Material and Methods". Similar data were obtained with DLD-1, HT-29, HCT-15 and SW-480.

TABLE II – INHIBITION OF COLON CARCINOMA CELL PROLIFERATION BY REPEATED TREATMENTS WITH rIFN-β (50 U/ml)

| Cell line | % Inhibition[1] | | |
| | Number of treatments | | |
	One[2]	Two[3]	Four[4]
Colon carcinoma			
SW-620	41	68	>99
SW-480	55	68	>99
DLD-1	76	82	>99
HCT-15	86	88	>99
HT-29	62	72	>99
Lung fibroblast			
WI-38	0	0	0

[1]Inhibition was calculated on day 8.–[2]Treated with a single dose of rIFN-β on day 0.–[3]Treated with rIFN-β on day 0 and day 4.–[4]Treated with rIFN-β on day 0 and days 2, 4 and 6.

effect, which was ablated in the presence of anti-IFN-β antibody. Control IgG had no detectable effect on the inhibitory action of rIFN-β.

The growth-inhibitory activities of rIFN-α and rIFN-γ were also investigated. Highly purified rIFN-α, representing a consensus sequence of several IFN-α sequences (rIFN-α Con₁), was ineffective against 5 colorectal cell lines tested (Fig. 5a), even at concentrations up to 10,000 U/ml (data not shown). rIFN-γ at 8 U/ml blocked HT-29 proliferation by 90% but displayed only modest (HCT-15) or minimal (DLD-1, SW620 and SW-480) inhibitory activity against other colon cell lines (Figure 5b).

DISCUSSION

The results of the present study have revealed that rIFN-β is significantly more potent than either rIFN-α or IFN-γ in blocking the proliferation of a number of colon carcinoma cell lines in vitro. rIFN-β inhibited the growth of 18/18 colon cell lines in monolayer culture and 8/9 cell lines in soft agar. In contrast, rIFN-γ displayed activity against only 2/5 cell lines in monolayer assay while rIFN-α had no inhibitory activity on 5 colorectal cell lines tested.

The anti-proliferative activity of rIFN-β was both dose- and time-dependent. Inhibitory activity was seen at day 3 following
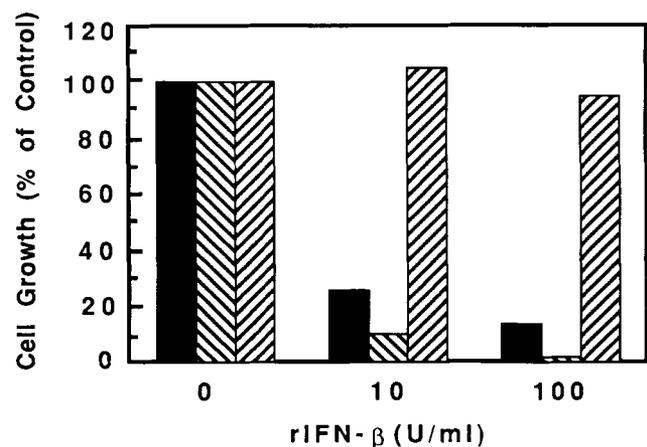

FIGURE 4 – Neutralization of rIFN-β growth inhibitory activity by anti-IFN-β antibodies. HCT-15 colorectal carcinoma cells in monolayer culture were challenged with 10 or 100 U/ml of rIFN-β in the absence (■) or the presence of 3,000 neutralizing units of anti-IFN-β (▨), or control (▧) immunoglobulins. Growth inhibition was quantitated using image analysis as described in "Material and Methods". The results are representative of 2 experiments. Similar data were obtained with SW-620, DLD-1, HT-29 and SW-480.
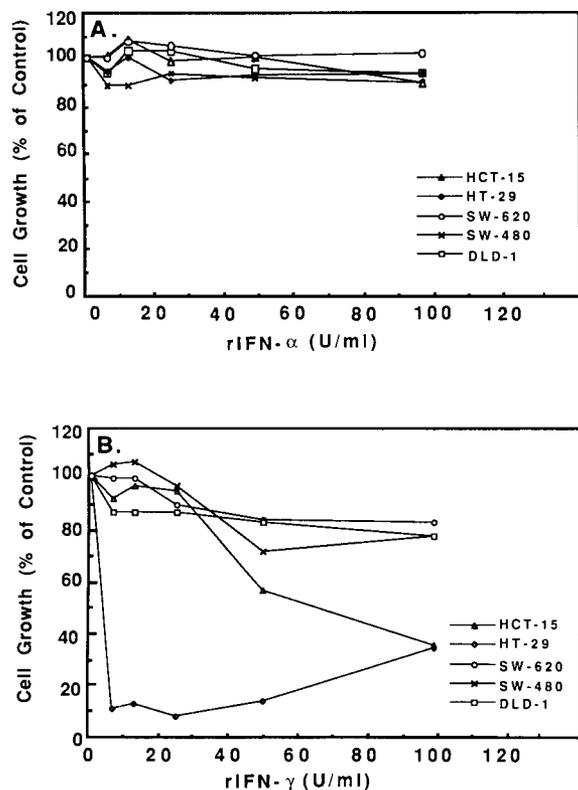
FIGURE 5 – Growth inhibitory activity of rIFN-α and rIFNγ on human colon carcinoma cells. rIFN-α (a) and rIFN-γ (b) were incubated with the indicated colorectal carcinoma lines in the monolayer assay as described in "Material and Methods" and growth inhibition quantitated by image analysis. Colorectal cell lines are HT-29, HCT-15, SW-480, DLD-1 and SW-620.

rIFN-β treatment and maximal inhibition was obtained at 50–100 U/ml. Repeated challenge with rIFN-β over the 8-day assay period enhanced its anti-proliferative activity. This is probably due to the limited half-life of rIFN-β in conditioned medium, and confirms that optimal inhibition is best achieved by maintaining effective rIFN-β concentrations for prolonged periods (days). rIFN-β failed to block fibroblast proliferation and its inhibitory activity towards colorectal carcinoma cells was neutralized by anti-IFN-β antibodies, demonstrating that its cytostatic activity was selective and not attributable to non-specific toxicities.

The reasons for rIFN-β's greater potency compared with rIFN-α and rIFN-γ have not been addressed in the present study. The responsiveness of SW-620, SW-480, DLD-1 colorectal cell lines to IFN-γ anti-proliferative effect is in agreement with earlier work (Pfizenmaier et al., 1985). Other investigations have reported analogous gradations in the activity of different interferons towards transformed and neoplastic

cells (Borden et al., 1982; Nagai and Arai, 1982; Czarniecki et al., 1984; Schiller et al., 1986), but the mechanisms underlying these discriminatory activities remain unclear. Particularly puzzling is the contrasting activities of IFN-α and IFN-β, since both these lymphokines, although sharing limited (30%) homology, apparently bind to the same receptor in human cells (Rubinstein and Orchansky, 1986). However, other workers have also reported that IFN-β and IFN-α subtypes display divergent activity profiles in a number of biological systems including antiviral and anti-proliferative potency (Nagai and Arai, 1982; Rehberg et al., 1982), NK activity (Ortaldo et al., 1983), and modulation of cell surface receptor expression (Giacomini et al., 1984; Hokland, 1985). The reasons for this are unknown but may reflect the distinct abilities of IFN-α and IFN-β to induce receptor activation and mobilize similar or distinct signal transduction mechanisms.

The tumorigenic, metastatic and morphological properties of colon carcinoma cell lines used in the current investigation have been reported by us in detail (Trainer et al., 1988), but there was no detectable association between any of these parameters and sensitivity (or refractoriness) to the interferons. For example, while HT-29 cells are highly tumorigenic in nude mice, SKCO-1 cells, under identical conditions, failed to form detectable neoplasms, yet both lines are sensitive to rIFN-β. Similarly, we failed to detect any obvious correlations between responsiveness to rIFN-β and cell doubling time in vitro, production of tumor-associated markers (CEA, CA19-9), xenograft histology or expression of specific oncogenes (Trainer et al., 1988).

Clinical trials with different types of interferon have yielded disappointing results (Silgals et al., 1984; Eggermont et al., 1985; Krown et al., 1987; McPherson and Tan, 1980; Abdi et al., 1986). IFN-α is ineffective against the solid malignancies while naturally produced IFN-β, although not as extensively studied as rIFN-α, has failed to elicit encouraging responses in patients with colorectal tumors (McPherson and Tan, 1980; Abdi et al., 1986). However, there are reports, albeit rare, of significant responses to rIFN-β (Hawkins et al., 1985; Schiller et al., 1987b; Lillis et al., 1987; Triozzi et al., 1987), suggesting that certain patients may benefit from advances in interferon therapy. At the same time, it is also clear that the cytostatic effect of rIFN-β against cultured colorectal carcinoma cells is a poor predictor of clinical activity. There are probably many reasons for this, ranging from the inappropriateness of highly selected cell lines for studies on growth regulation to poor pharmacokinetics and pharmacodistribution of rIFN-β in vivo. Nevertheless, the breadth and potency of rIFN-β in blocking the proliferation of an extensive panel of colorectal carcinoma cells in vitro suggest that further investigations on its mechanism of action may provide better insight into the factors that influence colon tumor cell proliferation.

ACKNOWLEDGEMENTS

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