

Cytokinetic Effects of Interferon in Colorectal Cancer Tumors: Implications in the Design of the Interferon/5-Fluorouracil Combinations

Stefano Cascinu,¹ Elena Del Ferro,² Anna Fedeli, Cesare Grianti, Giampaolo Foglietti, Quinto Olivieri, Marcello Acetosio, Marcello Bartolucci, Vittorio Saba, Ferdinando Lungarotti, and Giuseppina Catalano

Servizio di Oncologia [S. C., E. D. F., A. F., G. C.], Laboratorio Analisi [C. G., M. A., M. B.], and Divisione di Chirurgia [G. F., F. L.], Ospedali Riuniti, P. le Cinelli 4, 61100 Pesaro, and Cattedra di Semeiotica Chirurgica, Università di Ancona, Ancona [Q. O., V. S.], Italy

ABSTRACT

Interferon (IFN) has been shown to enhance the cytotoxic effects of 5-fluorouracil (5FUra) in colorectal cancer, and clinical trials with this combination resulted in higher response rate with respect to 5FUra alone. IFN is generally administered s.c. three times a week. This prolonged exposure could determine a block of tumor cells in the G₀-G₁ phase of the cell cycle, thus rendering tumor cells insensitive to 5FUra, an S-phase specific agent. In order to verify the presence of this block, 21 operable colorectal cancer patients were treated with IFN- α_{2b} at the dose of 3 megaunits every other day in the week before operation, while another 22 represented the control group. Samples of tumor tissue were taken at endoscopy and operation. [³H]Thymidine labeling index and flow cytometry were used to assess the S-phase fraction. In IFN treated patients, we found a significant statistical difference between the mean percentage of S-phase fractions evaluated either by labeling index ($P = 0.00001$) or by flow cytometry ($P < 0.001$). On the contrary, this difference was not present in the control group: labeling index, $P = 0.06$; flow cytometry, $P = 0.08$. Furthermore a significant increase in the G₀-G₁ phase of the cell cycle was found after IFN administration ($P < 0.001$) but not in the control group. Our results suggest that IFN reduces the S-phase fraction in colorectal cancer tumors. This action should be considered in the design of the 5FUra/IFN combination because it could decrease 5FUra activity, leading to a loss or a decrease in the advantage of 5FUra modulation by IFN.

INTRODUCTION

IFN³ has been shown to synergistically enhance the cytotoxic effects of 5FUra *in vitro* (1). On the basis of these data, several clinical trials with 5FUra and IFN were initiated, resulting in objective clinical response rates higher than those expected with 5FUra alone (2-5).

However, the mechanism of interaction between these agents and optimal doses and schedule has not been identified yet. Knowledge of these aspects may have the potential to lead to a more effective regimen.

Apart from doses and sequence of IFN/5FUra administration another critical point could be the duration of IFN exposure. Considering in fact the reported capability of IFN to inhibit *in vitro* the establishment of competence in G₀-S transition, prolonged IFN administration could not be the most convenient procedure. It could in fact determine a reduction of the S-phase fraction and thus render tumor cells insensitive to 5FUra, a S-phase specific agent (6-8).

In order to verify if this block of cell cycle by IFN is present in colorectal cancer patients given IFN, we analyzed the S-phase fraction in samples of tumor tissue from operable colorectal cancer patients before and after IFN administration, using the [³H]thymidine labeling index and flow cytometry.

Received 6/9/93; accepted 9/14/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom requests for reprints should be addressed, at Servizio di Oncologia, Ospedali Riuniti, P. le Cinelli 4, 61100 Pesaro, Italy.

² Recipient of a grant from Associazione Italiana Ricerca Cancro (AIRC).

³ The abbreviations used are: IFN, interferon; 5FUra, 5-fluorouracil; LI, labeling index; FC, flow cytometry; PI, propidium iodide.

PATIENTS AND METHODS

Patients. Patients with operable colorectal cancer were eligible for this study. Twenty-two colorectal cancer patients represented a control group in order to verify the presence of perturbations in cell kinetics induced by operation. The subsequent treatment, plan foresaw the administration of IFN- α_{2b} , at the dose of 3 million units every other day in the week before operation in at least 20 more colorectal cancer patients.

Patients had to have adequate hepatic, renal, cardiac, and bone marrow function. They were excluded for IFN treatment for active infection, severe coexisting disease, or inadequate nutrition. Patients were allowed to receive acetaminophen to reduce interferon induced fever, chills and myalgias but not nonsteroid antiinflammatory drugs or corticosteroids. In the same way patients who needed total parenteral nutrition were excluded from this study because it was demonstrated to modify cell kinetics in other tumors (9).

Patients were required to give informed consent. Both the consent and protocol were approved by the Institutional Review Board.

Specimen Sampling. Tumor tissue samples were taken at endoscopy for baseline evaluation and then at operation. According to endoscopists and surgeons the time of endoscopy and operation was practically the same (about 9 to 11 a.m.) to avoid possible alterations in the cell kinetics due to the well demonstrated circadian rhythm present in colorectal mucosa (10).

Four forceps biopsy specimens from different sites of the tumor were removed during colonoscopy for LI determination and cytometric examination. Immediately after surgical resection, four nonnecrotic sections of the tumor were sampled at the inner (luminal) border, in order to obtain samples, for LI determination and cytometric analysis, comparable to those obtained by endoscopy, as much as possible.

Determination of LI. The LI was determined on fresh tumor material. Within 20 to 30 min of the excision, tumor specimens were cut into fragments (usually 3 to 10 mm³). They were incubated in M199 medium containing 20% fetal calf serum, antibiotics (100 units/ml of penicillin and 100 μ g/ml of streptomycin), and 6 μ Ci/ml of tritiated thymidine (specific activity, 25 Ci/mmol) for 1 h with agitation at 37°C, fixed in formalin, and embedded in paraffin. The blocks were cut in 4- μ -thick sections, which were rehydrated and coated with liquid photographic emulsion (K-5; Ilford, London, United Kingdom) at 45°C. After an exposure time of 3 days at 4°C autoradiograms were developed in Phenisol (Ilford) for 5 min and fixed in Hypam (Ilford) for 10 min. The samples were stained with hematoxylin and eosin.

Cells in the S phase of the replicative cycle during the incubation period showed several (usually more than six) silver grains over the nucleus and were therefore considered "labeled." The LI was determined by scoring a total of 2000 to 3000 cells on the different fragments of each tumor. It was defined as the percentage ratio between labeled and total tumor cells (11).

Flow Cytometric Study. Single cell suspensions were prepared from 40- μ m-thick sections obtained from 43 formalin-fixed and paraffin-embedded blocks. Sections 4 μ m thick were also serially cut from the same tissue blocks and stained with hematoxylin and eosin to confirm the presence of tumor cells in the specimens. The 40- μ m-thick sections were treated with a standard procedure (Coulter DNA-prep Reagents Kit), according to the method of Bauer (12), with slight modifications to obtain nuclei suspensions. Briefly, they were dewaxed in xylene 3 times (1 h, 1 h, and overnight), rehydrated successively for 10 min through a series of alcohol concentrations (100, 95, 70, and 50%), and then washed twice with distilled water. The samples were agitated several times during rehydration. The sections were then treated for 30 min at 37°C in a 5-mg/ml solution of pepsin (Sigma) in 0.9% NaCl adjusted to pH 1.5 with 2 N HCl. The tubes were placed in a water bath with intermittent vortex mixing. The suspensions obtained were filtered through a 35 μ m pore nylon filter. Cell

count was made to ensure that a range of 3 to 10×10^6 cells/ml for sample was present. The suspensions were then centrifuged and the pellet was stained with PI at a concentration of $50 \mu\text{g/ml}$ in H_2O together with RNase (type III-A, from bovine pancreas), 0.1% NaN_3 , saline, and stabilizers. The samples were analyzed 30 min to 3 hours after addition of PI. Flow cytometric analysis was carried out with an Epics Profile II flow cytometer (Coulter Electronics, Hialeah, FL), equipped with a 1.5-W argon ion laser (Coherent, Palo Alto, CA). The fluorescence of PI stained nuclei, excited at 488 nm with 200 mW of light regulated power, was quantitated after passing through a 457 to 502 nm blocking filter. Histograms were recorded for a minimum of $25,000$ nuclei (excluding internal standard) at flow rates no greater than 30 to 50 events/s. Isoton was used as the sheath fluid. Data obtained as 1024 -channel histograms were identical to those with 256 channels of resolution. Electronic gating of the light scatter signal was used to exclude cellular debris and aggregates from data acquisition. Calibration microspheres (DNA Check, grade I; Fine Particle Division, Coulter) were used for daily instrument alignment. The analyzed samples were regarded as diploid, when they exhibited a single $\text{G}_0\text{-G}_1$ peak; in the case of two discrete $\text{G}_0\text{-G}_1$ peaks samples were judged aneuploid. The S-phase fraction, determined as the area under the curve between the $\text{G}_0\text{-G}_1$ and $\text{G}_2\text{-M}$ peaks, was calculated using Multicycle Cell Cycle Analysis Software program (Phoenix Flow System, Inc.). For aneuploid tumors, the S-phase fraction was calculated for each population, as far as possible.

The personnel involved in the experiment did not know if the samples of tumor tissue were treated with IFN or not. Student's t test was performed to assess potentially significant differences between individual groups of observations. The test statistics were then compared with values obtained from standard two tailed tables. $P < 5\%$ was accepted as indicating probable significance when comparing the various group (13).

RESULTS

Twenty-two colorectal cancer patients were included in this study as control group while 21 received IFN at the dose and schedule planned. Patient characteristics of both groups are summarized in Table 1. The results obtained with FC and LI are shown in Tables 2 and 3. Cellular populations of two groups, IFN treated patients and controls, did not differ in terms of cell cycle distribution and other kinetic properties at baseline. All the tissue samples were evaluable for flow cytometric analysis. In seven patients of the control group and in three IFN treated patients, bioptic specimens were not sufficient for a correct evaluation of LI because of the small number of tumor cells.

Table 1 Patient characteristics

	Control group	IFN treated patients
No. of patients	22	21
Age, median (range)	68 (50-79)	66 (32-78)
Colon/rectum	14/8	13/8
Histological type		
Well differentiated	9	8
Moderately differentiated	12	12
Poorly differentiated	1	1
Stage (Dukes)		
A	2	1
B	16	15
C	4	5

Table 2 Labeling index: results

	% of S-phase fraction		P
	Endoscopy	Operation	
Control group (15) ^a	9.13 ± 3.73^b	7.38 ± 2.72	0.06
IFN treated (18) ^a	13.75 ± 5.2	3.82 ± 3.67	0.00001

^a Evaluable patients.

^b Mean \pm SE.

Table 3 Flow cytometry: cell cycle distribution (mean % \pm SE)

	Control group (22) ^a		IFN treated (21) ^a		P
	E	O	E	O	
$\text{G}_0\text{-G}_1$	72.48 ± 14	73.4 ± 10.4	69.82 ± 10.4	78.64 ± 7.9	<0.001
S	19.17 ± 6.9	19.78 ± 8.2	24.87 ± 9.53	16.66 ± 7.4	<0.001
$\text{G}_2\text{-M}$	8.3 ± 12.4	6.79 ± 7.7	5.3 ± 5.94	4.7 ± 6.15	NS

^a Evaluable patients; E, endoscopy; O, operation; NS, not statistically significant.

Table 4 Control group percentage of S-phase fraction according to stage (Dukes) and tumor differentiation

	% of S-phase fraction	
	Endoscopy	Operation
	Stage B	19.33 ± 8.62
Stage C	18.83 ± 1.64	18.62 ± 7.35
G_1^a	19.25 ± 6.43	23.02 ± 9.08
G_2	19.64 ± 8.33	18.79 ± 7.18

^a G_1 , well differentiated; G_2 , moderately differentiated.

Table 5 IFN treated patients: percentage of S-phase fraction according to stage (Dukes) and tumor differentiation

	% of S phase fraction		P
	Endoscopy	Operation	
	Stage B	21.27 ± 6.40	
Stage C	31.51 ± 11.7	19.18 ± 8.48	<0.01
G_1^a	22.24 ± 7.18	15.52 ± 7.24	<0.05
G_2	27.11 ± 10.3	17.37 ± 7.76	<0.01

^a G_1 , well differentiated; G_2 , moderately differentiated.

There is a significant statistical difference between the mean percentage of the S-phase fractions in IFN treated patients evaluated either by LI ($P = 0.00001$) or FC ($P < 0.001$). On the contrary, this difference is not present in the control group (LI, $P = 0.06$; FC, $P = 0.08$). At the same time, we found a significant increase in the percentage of $\text{G}_0\text{-G}_1$ after IFN administration ($P < 0.001$) but not in the control group (Table 3).

We reported also the results of the cell cycle distribution, determined by FC, considering stage and tumor differentiation. As shown in Tables 4 and 5 no difference was found in comparison with overall results. In the IFN treated Dukes C patients, the mean percentage of the S-phase fractions obtained from the endoscopy specimens was much higher than that in the control group, because three of these five tumors presented the highest values of the S-phase fraction. These values were substantially decreased after IFN treatment, confirming the cytokinetic effects of IFN.

No patient presented side effects due to IFN treatment, nor was any operation delayed or complicated by treatment.

DISCUSSION

Potential mechanisms of interaction between IFN and 5FUra remain unclear and may result from effects at the cellular level or from augmented pharmacokinetic effects (14, 15). This incompletely defined mechanism of interaction between 5FUra and IFN caused an empiric design of clinical regimens. In fact, in most of 5FUra/IFN combinations, while the 5FUra weekly administration is practical and a commonly used mode of administration, the dose and schedule of IFN represent a regimen that approaches the maximum tolerated dose. However, no antecedent information allows one to predict that these agents, combined in these doses and schedules, interact optimally.

Knowledge of these aspects may have the potential to lead to a more effective regimen.

Some aspects of these problems are now partly clarified. Intermediate doses of IFN seem to be more effective than high doses or low doses (16, 17). Furthermore, preclinical evidence suggests that IFN should be given concurrently with 5FUra for optimal potentiation (1).

Another important critical point in the 5FUra/IFN combination could be determined by one of the predominant cellular effects of IFN. It was demonstrated to be able in fact to determine the inhibition of cell cycle progression with partial block in the transition from G_0 - G_1 to S phase in several normal and tumor cell lines (6-8). Furthermore, Stolfi *et al.* (18) demonstrated in mice the presence of a transient period (about 42 h) of inhibition of bone marrow cell cycling following the administration of IFN or IFN inducer, with an accumulation of marrow cells in G_1 and the reduction in the percentage of cells in S phase. They suggested that, in consideration of the pharmacological properties of 5FUra, an S-phase specific agent, this IFN action could result in a reduction of 5FUra-induced toxicity. In fact, if the tumor cells are not susceptible or are less susceptible to the antiproliferative action of IFN compared to normal host tissue, the coadministration of IFN with 5FUra could permit the use of higher doses of 5FUra than normally tolerated, with a resultant increase in the therapeutic efficacy of 5FUra.

Unfortunately, Stolfi *et al.* did not evaluate the cytokinetic effects of IFN in tumor cells. There is the possibility that IFN does not exert differential antiproliferative activity in normal *versus* malignant cells, with the danger that the previous administration of IFN may diminish the anticancer activity of 5FUra rather than increasing activity through biochemical or pharmacokinetic modulation.

In clinical practice this could be of importance. In fact, most of the IFN/5FUra regimens foresee prolonged administration of IFN (*i.e.*, three times a week) even when 5FUra is not administered. This prolonged IFN administration could determine a persistent block of tumor cells in G_0 - G_1 , thus reducing the advantage of the modulation of 5FUra. Because of this possible clinical relevance, we investigated the interaction between IFN and cell kinetics in operable colorectal cancer patients, receiving IFN- α_{2b} at the dose of 3 million units, every other day in the week before operation. This schedule was chosen because the three times/week administration of IFN is very common, while the low dose should have avoided the possible evidence of side effects that could delay operation.

Our findings seem to suggest that IFN is able to reduce the S-phase fraction, determining a block of tumor cells in G_0 - G_1 . This evidence was confirmed by two different analyses. Both LI and FC showed a statistical significant reduction of the S-phase fraction and an increase of tumor cells in G_0 - G_1 . One of the possible objections is that intratumoral heterogeneity can be responsible for observed differences in S-phase fraction, as observed by some investigators in individual tumors sampled at multiple sites (19, 20). However, according to other observations (21, 22) it seems unlikely that the observed decreases in S-phase fraction shown in our study by LI and FC result solely from the samples of heterogeneous populations. Each tumor serially sampled demonstrated a decrease in S-phase fraction. The likelihood of committing a type I statistical error by an inadvertent sampling of unrepresentative populations is lowered by the consistency of the decrease in all tumor samples, as already suggested by Frank *et al.* (9). Moreover, to evaluate the possible alterations of cell kinetics induced by operation, we studied tumor cell kinetics in 22 operable colorectal cancer patients not treated with IFN. No significant difference in S-phase fraction was present before and after operation in this group confirming that cytokinetic alterations seen in patients treated with IFN are due to IFN exposure and not to operation.

Indirectly, evidence of this effect could be derived also from the results of studies analyzing the best sequence of IFN/5FUra administration. It was found that 5FUra cytotoxicity was enhanced by concomitant or subsequent exposition to IFN, while the reverse sequence, IFN followed by 5FUra, abrogated the cytotoxic effect of 5FUra suggesting that pretreatment with IFN could protect tumor cells (23).

However, apart from data on inhibition of malignant cell growth (6-8) and the results reported by Stolfi *et al.* (18) on normal bone marrow cells in mice, we are aware of another work only on the modifications of cell kinetics induced by IFN in colorectal cancer cells. This study concluded in opposite way respect to our results. Wadler *et al.* showed that neither IFN- α_{2a} nor IFN- γ had any significant cytokinetic effect on two colorectal cancer cell lines tested, whereas IFN- β markedly increased the proportion of cells in both S and G_2 (24).

Considering the possible importance of our results for clinical practice and, on the other hand, the paucity of data present in literature we think that further studies are needed to determine the significance of the cytokinetic effects induced by IFN, in order to define the optimal timing of 5FUra and IFN. The block of tumor cells in G_0 - G_1 phase in fact, as demonstrated by our study, could determine a decrease in the advantage of 5FUra modulation by IFN. Cyclic rather than continuous IFN administration could reduce the consequence of the alteration of cell kinetics, maintaining at the same time the advantage of biochemical and/or pharmacokinetic modulation.

REFERENCES

- Wadler, S., and Schwartz, E. L. Antineoplastic activity of the combination of interferon and cytotoxic agents against experimental and human malignancies: a review. *Cancer Res.*, *50*: 3473-3486, 1990.
- Wadler, S., Schwartz, E. L., Goldman, M., Lyver, A., Rader, M., Zimmerman, M., Itri, L., Weinberg, V., and Wiernik, P. H. Fluorouracil and recombinant alfa-2a-interferon: an active regimen against advanced colorectal carcinoma. *J. Clin. Oncol.*, *7*: 1769-1775, 1989.
- Pazdur, R., Ajani, J. A., Patt, Y. Z., Winn, R., Jackson, D., Shepard, B., DuBrow, R., Campos, L., Quaraishi, M., Faintuh, J., Abruzzese, J. L., Gutterman, J., and Levin, B. Phase II study of fluorouracil and recombinant interferon alfa-2a in previously untreated advanced colorectal carcinoma. *J. Clin. Oncol.*, *8*: 2027-2031, 1990.
- Kemeny, N., Younes, A., Seiter, K., Kelsen, D., Sammarco, P., Adams, L., Derby, S., Murray, P., and Houston, C. Interferon α -2a and 5-fluorouracil for advanced colorectal carcinoma. *Cancer (Phila.)*, *66*: 2470-2475, 1990.
- Wadler, S., Lembersky, B., Atkins, M., Kirkwood, J., and Petrelli, N. Phase II trial of fluorouracil and recombinant interferon alfa-2a in patients with advanced colorectal carcinoma: an Eastern Cooperative Oncology Group Study. *J. Clin. Oncol.*, *9*: 1806-1810, 1991.
- Lin, S. L., Kiguchi, T., Pledger, W. J., and Tamm, I. Interferon inhibits the establishment of competence in G_0 / G_1 -phase transition. *Science (Washington DC)*, *233*: 356-359, 1986.
- Balkwill, F. R. Interferons. *Lancet*, *2*: 1060-1063, 1990.
- Revel, M. The interferon system in man: nature of the interferon molecules and mode of action. *In*: Y. Becker (ed.), *Antiviral Drugs and Interferon*, pp. 358-433. New York: Martinus Nijhoff, 1984.
- Frank, J. L., Lawrence, W., Banks, W. L., McKinnon, J. G., Chan, W. M., and Collins, J. M. Modulation of cell cycle kinetics in human cancer with total parenteral nutrition. *Cancer (Phila.)*, *69*: 1858-1864, 1992.
- Buchi, K. N., Moore, J. G., Hrushesky, W. J. M., Sothorn, R. B., and Rubin, N. H. Circadian rhythm of cellular proliferation in the human rectal mucosa. *Gastroenterology*, *101*: 410-415, 1991.
- Silvestrini, R., Daidone, M. G., and Gasparini, G. Cell Kinetics as a persistent prognostic marker in node-negative breast cancer. *Cancer (Phila.)*, *56*: 1982-1988, 1985.
- Bauer, K. D. DNA content analysis. *In*: Seventh Annual Course in Clinical Flow Cytometry, Nashville, TN, November 5-9, 1990.
- Glantz, S. A. *Primer of Biostatistics*, Ed. 2. New York: McGraw-Hill Book Co., 1987.
- Schwartz, E. L., Hoffman, M., O'Connor, C., and Wadler, S. Stimulation of 5-fluorouracil metabolic activation by interferon- α in human colon carcinoma cells. *Biochem. Biophys. Res. Commun.*, *182*: 1232-1239, 1991.
- Schuller, J., Czejka, M., Miksche, M., Fogl, U., and Scherthaner, G. Influence of interferon alfa-2b (IFN) plus or minus leucovorin (LV) on pharmacokinetics (PK) of 5-fluorouracil. *Proc. Am. Soc. Clin. Oncol.*, *10*: 98, 1991.
- Wadler, S., Goldman, M., Lyver, A., and Wiernik, P. H. Phase I trial of 5-fluorouracil and recombinant alfa 2a-interferon in patients with advanced colorectal carcinoma. *Cancer Res.*, *50*: 2056-2059, 1990.
- Grem, J. L., McAtee, N., Murphy, R. F., Balis, F. M., Steiberg, S. M., Hamilton, J. M.,

- Sorensen, J. M., Sartor, O., Kramer, B. S., Goldstein, L. J., Gay, L. M., Caubo, K. M., Goldspiel, B., and Allegra, C. J. A pilot study of interferon alfa-2a in combination with fluorouracil plus high-dose leucovorin in metastatic gastrointestinal carcinoma. *J. Clin. Oncol.*, *9*: 1811–1820, 1991.
18. Stolfi, R. L., Martin, D. S., Sawyer, R. C., and Spiegelman, S. Modulation of 5-fluorouracil-induced toxicity in mice with interferon or with the interferon inducer, polyinosinic-polycytidylic acid. *Cancer Res.*, *43*: 561–566, 1983.
19. Carey, F. A., Lamb, D., and Bird, C. Intratumoral heterogeneity of DNA content in lung cancer. *Cancer (Phila.)*, *65*: 2266–2269, 1990.
20. Ljungberg, B., Stenling, R., and Roos, G. DNA content in renal cell carcinoma with reference to tumor heterogeneity. *Cancer (Phila.)*, *56*: 503–508, 1985.
21. Askensten, U. G., von Rosen, A. K., and Nilsson, R. S. Intratumoral variations in DNA distribution patterns in mammary adenocarcinomas. *Cytometry*, *10*: 326–333, 1988.
22. Kokal, W. A., Gardine, R. L., and Sheibani, K. Tumor DNA content in resectable, primary colorectal carcinoma. *Ann. Surg.*, *209*: 188–193, 1989.
23. Wadler, S., Schwartz, E. L., Goldman, M., Lyver, A., Itri, L., and Wiernik, P. H. Preclinical and clinical studies of 5-fluorouracil (FURA) and recombinant α -2a interferon (IFN) against gastrointestinal (GI) malignancies. *Clin. Res.*, *36*: 803, 1988.
24. Wadler, S., Wersto, R., Weinberg, V., Thompson, D., and Schwartz, E. L. Interaction of fluorouracil and interferon in human colon cancer cell lines: cytotoxic and cytokinetic effects. *Cancer Res.*, *50*: 5735–5739, 1990.