Effect of the Combined Treatment with 5-Fluorouracil, γ-Interferon or Folinic Acid on Carcinoembryonic Antigen Expression in Colon Cancer Cells

Angelo Aquino, Salvatore P. Prete, John W. Greiner, Anna Giuliani, Grazia Graziani, Mario Turriziani, Rosaria De Filippi, Giovanna Masci, Enzo Bonmassar, and Liana De Vecchis

Department of Neuroscience, Section of Pharmacology and Medical Oncology University of Rome, “Tor Vergata,” 00133 Rome, Italy [A. A., S. P. P., A. G., G. G., M. T., G. M., E. B., L. D. V.]; Laboratory of Tumor Immunology and Biology, National Cancer Institute, NIH, Bethesda, Maryland 20892 [J. W. G.]; Istituto Dermopatico dell’Immacolata, 00167 Rome, Italy [E. B.]; Department of Pathology and Experimental and Clinical Medicine University of Udine, 33100 Udine, Italy [R. D. F.]; and Institute of Experimental Dermopatico dell’Immacolata, 00167 Rome, Italy [E. B.; Department of Neuroscience, Section of Pharmacology and Medical Oncology University of Rome, “Tor Vergata,” 00133 Rome, Italy [A. A.]

ABSTRACT

5-Fluorouracil (5-FU) and human recombinant γ-interferon (γ-IFN) were found to increase the expression of carcinoembryonic antigen (CEA) in human cancer cells in vitro. In the present study, the antimitabolite was associated with γ-IFN or folic acid (FA), a biochemical modulator of cellular metabolism of 5-FU, able to increase its antineoplastic activity. Treatment of two human colon cancer cell lines (HT-29 and WiDr) with 5-FU + γ-IFN resulted in an increase of CEA expression higher than that obtainable with both agents alone, although no synergistic effects were obtained. This was demonstrated in terms of: (a) mRNA transcripts (HT-29); (b) cytoplasm and membrane CEA protein levels detected by Western blot analysis (HT-29); and (c) plasma membrane reactivity determined by flow cytometry analysis (HT-29 and WiDr). Moreover, 5-FU + γ-IFN increased HLA class I molecules in the HT-29 cell membrane over that obtainable with γ-IFN alone. In contrast, both agents did not induce the expression of the costimulatory molecule B7-1. Treatment with FA enhanced the antitumor effect of 5-FU but not its ability to augment CEA expression. This suggests that the FA-sensitive biochemical mechanism of action of 5-FU is not involved in its effect on CEA expression. In vivo studies showed, for the first time, that 5-FU, alone or combined with γ-IFN, increases the amount of CEA protein over controls, either in cancer cells or in peripheral blood of nude mice bearing HT-29 cells. These results could be of potential diagnostic and/or therapeutic value when CEA protein is the target of humoral or cell-mediated immunity.

INTRODUCTION

Among antineoplastic agents, 5-FU remains the most effective agent for colorectal cancer. The antimitabolite exerts its cytotoxic activity mainly through inhibition of TS, which leads to depletion of TTP necessary for DNA synthesis. However, clinical trials conducted for more than 30 years do not indicate that this agent can provide satisfactory therapeutic results (i.e., 15–20% total response rate). For this reason, several attempts have been made to improve the antitumor activity of 5-FU through the association with other agents, such as FA (1, 2) or IFNs (3, 4). It was found that both FA and IFNs are capable of increasing the antitumor activity of 5-FU through different mechanisms. In fact, FA increases intracellular reduced folate, which forms together with 5-fluoro-dUMP (FdUMP) a stable ternary complex with TS, thus producing a permanent inhibition of the enzyme (5). α-IFN augments the effect of 5-FU by increasing the DNA damage, without enhancing FdUMP levels (6). It has also been reported that γ-IFN reduces the de novo biosynthesis of TS, elicited by cell exposure to 5-FU (7). However, Van der Wilt et al. (6) did not confirm these results, leaving open the question of the actual mechanism underlying the combined antitumor effects of γ-IFN + 5-FU.

CEA, a M. 180,000–200,000 glycoprotein, is a widely used human tumor marker for various types of neoplasias, including gastrointestinal, breast, and lung cancer. Studies performed originally by Maas et al. (8) and confirmed later in our laboratory (9, 10) have shown that treatment of several colon cancer cell lines with 5-FU in vitro is followed by increased expression of membrane-associated CEA. Further investigations have established that IFNs (α, β, and γ) can augment the expression of several human tumor-associated antigens (11). In particular, γ-IFN is able to increase CEA and MHC antigens expression on...
tumor cells (12, 13). This finding is of potential clinical interest, in view of the possible role that can be played by CEA in a number of diagnostic and treatment modalities. Actually, CEA can be involved in two main immunotherapeutic approaches, i.e., targeting of anticancer agents or radionuclides by tumor-selective anti-CEA monoclonal antibodies (14–16) and new anti-CEA antitumor vaccines, eliciting MHC-restricted immune responses against CEA-derived peptides (17–21).

On these bases, it was decided to explore the influence of γ-IFN or FA on the increase of CEA expression, mediated by 5-FU. The data, illustrated in the present report, show that in vitro as well as in vivo treatment with γ-IFN + 5-FU resulted in CEA levels higher than those induced by the two agents alone. On the other hand, FA did not influence the expression of this tumor marker nor modify the effect of 5-FU on CEA levels in HT-29 colon cancer cells.

MATERIALS AND METHODS

Drugs. γ-IFN was generously provided by Biogen Research Corp. (Cambridge, MA) and by Dr. G. Garotta (Hoffman-La-Roche, Basel, Switzerland); 5-FU (Roche, Milan, Italy) and FA (Sigma Chemical Co., St. Louis, MO) were available commercially.

mAbs. Expression of CEA, HLA class I, and costimulatory molecule B7-1 antigens was tested using the following mAbs: COL-1, an anti-CEA mAb (IgG2a), which was prepared, purified, and characterized in our laboratory as described previously (22); W6/32, an IgG2a mAb, able to recognize a monomorphic determinant of HLA class I antigen, which was obtained from Dako (Dakopatts, Copenhagen, Denmark); and the phycocerythrin-conjugated anti-CD80 mAb (IgG1: Becton Dickinson, Mountain View, CA), which recognizes the B7-1 co-stimulatory molecule. The FITC-conjugated F(ab')2 rabbit anti-mouse IgG was obtained from Dako.

Cell Lines and Culture Conditions. The human colon cancer cell lines HT-29 (ATCC, HTB38) and WiDr (ATCC, CCL218) were routinely grown in DMEM, supplemented with 2 mm glutamine, 1% (v/v) nonessential amino acids (100× solution), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS, hereafter referred to as CM. Adherent cells were removed using trypsin-EDTA solution, washed twice in PBS, and fresh CM was added to the cell monolayer in each flask. γ-IFN was also added on day 3, by multiple washings with PBS, and fresh CM was added to the flask at the desired concentration. In the experiments performed with FA, on day 3 after seeding, tumor cells were exposed to the agent at 37°C for 30 min, followed by 1-h incubation in medium alone or in medium containing 5-FU. The drugs were removed by multiple washings with PBS, and fresh CM was added to the cell monolayer in each flask. γ-IFN was also added on day 3, but it was maintained in culture until the end of the experiment. On day 6 after seeding (i.e., on day 3 after drug treatment), cells were counted and subjected to FACS (HT-29 and WiDr cells) and Northern and Western blot (HT-29 cells) analysis.

FACS Analysis. Flow cytometry analysis of membrane immunofluorescence was performed as follows. Cells were harvested with trypsin-EDTA solution, washed twice in PBS containing 0.02% sodium azide, and distributed into 3-ml tubes (10⁶ cells/tube). The cells were incubated with an excess of the primary mAb in an ice bath for 30 min, followed by two washes in PBS containing sodium azide. A 1:10 dilution of FITC-conjugated F(ab')2 rabbit anti-mouse IgG (second antibody) was then added to cell suspensions. The cells were again incubated in an ice bath for 30 min, washed twice in PBS, and analyzed using a FACSScan (Becton Dickinson). The percent of fluorescence intensity of 10,000 cells was recorded, and the background control (i.e., fluorescence obtained after incubation of the cells with the second antibody only) of individual samples was subtracted. The extent of CEA, HLA class I, and B7-1 antigen expression was calculated as percentages of positive cells and MFVs. Data analysis was performed by using "Consort 32" software on a Hewlett Packard computer (Hewlett Packard, Fort Collins, CO).

Cell Extracts. Cells were washed with PBS. The cell pellet was suspended in five volumes of lysis buffer [25 mm HEPES (pH 7.5), 2.5 mm MgCl₂, 2.5 mm EGTA, 50 mm 2-mercaptoethanol, 200 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mm phenylmethylsulfonyl fluoride, and 400 μg/ml soybean trypsin inhibitor], sonicated at 4°C for S s, and centrifuged at 15,000 X g for 1 h. The supernatant was collected and designated as the cytosol fraction. The pellet was resuspended in lysis buffer containing 1% Triton X-100, sonicated for 5 s, and centrifuged at 15,000 X g at 4°C in a microcentrifuge for 10 min. The supernatant was collected and defined as a membrane fraction. Membrane and cytosol fractions were heated in a boiling water bath for 2 min and separated in 10% SDS (w/v) polyacrylamide gels as described by Laemmli (23). All reagents were obtained from Sigma Chemical Co.

Immunoblotting. The method of Towbin et al. (24) was used for electrotransfer of proteins to nitrocellulose filters, using a Bio-Rad mini-blotting apparatus for electrophoresis (Bio-Rad, Hercules, CA). The transfer was carried out at 25 V overnight. After the transfer, membranes were incubated with 1% BSA in Tris-buffered saline [TBS: 20 mm Tris-HCl (pH 7.5) and 0.9% NaCl] with gentle agitation for 30 min. The membranes were then incubated at room temperature for 30 min, with COL-1 mAb diluted (14 μg/ml) in TBS containing 0.05% Tween 20 (TBST), washed twice with TBST, and incubated with alkaline phosphatase-coupled secondary antibody diluted 1:7500 in TBST for 30 min. The bands were visualized using the Protoblot (Promega Biotech, Madison, WI) color development system, as described by the manufacturer. Bidimensional densitometry of the immunoblot was performed using a Bio-Rad scanning apparatus (image densitometer, GS-670).

Northern Blot Analysis. Total RNA was extracted by the guanidinium thiocyanate method described by Chomczynski and Sacchi (25). Twenty μg of total RNA were denatured in 2.2 m formaldehyde and 50% formamide at 65°C and fractionated in 1.2% agarose gel containing 2.2 m formaldehyde. RNA was then transferred to Gene Screen Plus™ nylon membrane (DuPont NEN Research Products, Boston, MA) in 10× SSC (1× SSC = 0.15 M NaCl/0.01 M sodium citrate, pH 7.0/0.15 M sodium chloride/0.01 M sodium citrate). The membranes were prehybridized overnight at 65°C in 50% formamide and 2× SSC. The membranes were hybridized at 65°C in a temperature-controlled apparatus (imaging densitometer, GS-670).
0.1 M sodium chloride and 0.015 M sodium citrate). Prehybridization and hybridization were performed according to the manufacturer's instructions. Briefly, filters were prehybridized at 42°C in 50% formamide, 10% dextran sulfate, 1 M sodium chloride, and 1% SDS for 2 h. Hybridization was then performed at the same temperature in the prehybridization solution after addition of denatured salmon sperm DNA (100 µg/ml) and of the probe labeled with [α-32P]dCTP (3000 Ci/mmole; DuPont, Wilmington, DE), using a random primed labeling kit (Boehringer Mannheim, Indianapolis, IN). Filters were washed with 2X SSC at room temperature for 5 min, followed by washing in 2X SSC containing 1% SDS at 60°C for 30 min, and by a final wash in 0.1 X SSC at room temperature for 30 min. Autoradiography was performed at -80°C using Kodak XAR-5 films (Kodak, Rochester, NY).

cDNA Probes. Detection of CEA gene family members (BGP, CEA, and NCA) and CEA-specific transcripts was achieved using two different DNA probes. In the first instance, a 2.3-kb Smal fragment, encompassing the entire CEA cDNA (26), was isolated from the vector pGEM3Zf(+) (Promega, Madison, WI). Because of the high homology between CEA and BGP or NCA, this probe allows detection of 3.9, 3.7, and 1.8 kb (BGP transcripts), 3.5 kb (CEA transcript), and 3 kb (NCA transcripts). The specific CEA mRNA was detected using a 328-bp fragment from the 3'-untranslated region, a few nucleotides downstream from the Alu-type repetitive sequences (27). This probe was obtained by PCR amplification of 1 µg of genomic DNA extracted, according to standard procedures (28), from the HT-29 cell line, as described previously (10).

In Vitro Studies in Human Tumor Xenografts. Female BALB/c, athymic mice (20–25 g of body weight, 4–6 weeks of age) were obtained from the Frederick Cancer Research Facility (Frederick, MD). Mice were injected s.c. with 2 x 10⁶ HT-29 tumor cells in 200 µl of HBSS. After ~2 weeks, mice bearing progressively growing tumors (average tumor volume, 85 mm³) were selected.

5-FU was dissolved at a concentration of 5 mg/ml in sterile 0.9% saline. γ-IFN was diluted with sterile 0.9% saline to the appropriate concentration, and 200 µl were administered i.p. γ-IFN was tested periodically, and the antiviral titers remained unchanged for up to 6 months with storage at 4°C.

5-FU was injected i.p. at 15 mg/kg/day, whereas γ-IFN was administered i.p. at 10⁶ IU/day for 5 consecutive days. Untreated mice received the same volume (200 µl) of 0.9% saline.

Groups of mice (five animals/group) were treated with 5-FU (15 mg/kg), γ-IFN alone or in combination, as outlined above. Twenty-four h after the final treatment, all mice were sacrificed, and individual tumors weighed and pooled according to the appropriate treatment group. Sera were collected from individual mice. Tumor extracts were prepared as described (15) and were layered onto a discontinuous 20–40% (w/w) sucrose gradient and centrifuged at 25,000 × g for 17 h. Tissue that appeared as an opalescence band at the 20–40% interface was isolated, diluted with 5X Tris-HCl (0.1 M, pH 7.2), and centrifuged for 1 h as described. Membrane pellets of the untreated and the respective treatment groups were processed in PBS using a Teflon/glass homogenizer, and protein concentration was measured by the Lowry procedure. CEA levels in the isolated membranes as well as the individual serum samples were measured using the double-determinant RIA monoclonal in vitro test kit (Abbott Laboratories, Inc., Chicago, IL), according to the manufacturer’s instructions. Cutoff value for positive CEA concentrations was 5.0 ng/mg protein or higher. Membranes were diluted to 1.0 mg/ml when needed and assayed for CEA levels. In samples with CEA levels above the standard curve, the samples were diluted, and assay was repeated. All assays included internal low and high CEA standards.

RESULTS

Effect of 5-FU + γ-IFN on the Growth of HT-29 Cells and on CEA Expression of HT-29 and WiDr Cells in Vitro.

Cells of the HT-29 line were exposed to graded concentrations of 5-FU (i.e., from 7.8 to 500 µM), alone or in combination with γ-IFN (50 IU/ml) on day 3 after seeding, and tested for the number of viable cells (measured in terms of cells excluding trypan blue) on day 6. The results of a mean of three experiments, illustrated in Fig. 1, show that the antimitabolite induced a concentration-dependent decrease of the number of tumor cells. Limited inhibition of tumor growth was also obtained with γ-IFN alone. Moreover, tumor inhibition afforded by 5-FU (from 31 to 500 µM) + γ-IFN was higher than that obtainable with the cytokine or the antimitabolite alone, with additive but not synergistic antitumor effects.

Previous studies have shown that γ-IFN and 5-FU were capable of augmenting the expression of several human tumor-associated antigens, such as molecules of the CEA gene family (8–10, 29, 30). Therefore experiments, performed by FACS analysis were carried out to study the influence possibly afforded by the two agents, alone or in combination, on CEA expression. The antimitabolite increased CEA levels, and a linear relationship between the amount of 5-FU and CEA was detectable at concentrations ranging from 7.8 to 125 µM, although the baseline percentage of CEA-positive cells in unlabeled with the Alu-type repetitive sequences (27).
Drug-induced Increase of CEA Expression

Cytofluorimetric analysis of CEA expression in colon cancer cells exposed to 5-FU + γ-IFN. HT-29 (A) and WiDr (B) cells were exposed to 5-FU (31 μM) and γ-IFN (50 IU/ml) on day 3 after seeding and were tested by FACS analysis on day 6 by using COL-1 mAb. Y axis, relative number of cells; X axis, fluorescence intensity. Open curves, background fluorescence of cells incubated with FITC-conjugated F(ab)2, rabbit antimouse IgG (second antibody) alone. Filled curves, fluorescence of cells treated with the specific anti-CEA COL-1 mAb + second antibody. The percentages of CEA-positive cells (upper numbers) and MFV (lower numbers) are shown. C, percentage of CEA-positive HT-29 cells exposed to graded concentrations of 5-FU (ranging from 7.8 to 62.5 μM) alone or associated with graded concentrations of γ-IFN- (ranging from 4 to 500 IU/ml).

Effect of 5-FU + γ-IFN on CEA Protein Expression Evaluated by Western Blot Analysis. Determination of CEA protein on cell surface by FACS analysis could not reflect entirely the actual level of the tumor marker in the cell compartments. Actually, accessibility of the surface CEA epitope, recognized by COL-1 mAb, modulation of epitope immunoreactivity by neighbor molecules, or other unidentified factors, could play a role in the level of antigen positivity at the cell surface, detected in the experimental conditions, used for FACS analysis. Therefore, the expression of CEA protein on the cell membrane and in the cytoplasm was evaluated by Western blot analysis in denaturing conditions.

The results of immunoblot analysis (Fig. 3) show that treatment of HT-29 cells with graded concentrations (31.2–500 μM) of 5-FU is followed by an increase of the cytosolic and membrane-bound CEA expression. Combined treatment of HT-29 cells with 5-FU + γ-IFN induced an additional increase of CEA expression in both cytoplasmic and membrane fraction in comparison with the treatment with 5-FU or γ-IFN alone (Fig. 4A). Quantitation of the immunoblot by densitometric analysis (Fig. 4B) revealed that: (a) γ-IFN (50 IU/ml) or 5-FU (31 μM) induced a 4.6- and 2-fold increase, respectively, of cytosolic CEA in HT-29 cells compared with untreated cells, whereas the cytosol fraction of HT-29 cell treated with 5-FU + γ-IFN contained a 20-fold higher amount of CEA compared with untreated cells; (b) cell exposure to γ-IFN, 5-FU, or to the drug combination induced a 2-, 1.7-, and 3-fold increase, respectively, of membrane-bound CEA with respect to untreated cells; (c) drug combination (5-FU + γ-IFN) markedly increased...
were treated with 5-FU on day 3 after seeding and were extracted in hypotonic buffer on day 6. Cell homogenates were separated into membrane and cytosol fractions as described in “Materials and Methods.” Each fraction was solubilized and separated by SDS-PAGE, and CEA was visualized by immunoblotting with mAb COL-1.

the level of CEA in the membrane and cytosol fraction compared with the treatment with γ-IFN or 5-FU alone. Eighty to 90% of the total CEA was found in the membrane fraction of HT-29 cells, either untreated or treated with γ-IFN or 5-FU as single agents. Following combined treatment with 5-FU + γ-IFN, CEA increases relatively more in the cytoplasm than in the membrane fraction. In this case, HT-29 membranes contain 60% of the total CEA.

Effect of 5-FU and γ-IFN on CEA mRNA Expression. We have shown previously that, similarly to γ-IFN (31), the 5-FU-mediated increase of CEA protein expression in HT-29 cells is the result of enhanced gene transcription (10). In the present study, we have investigated whether the increase of CEA protein, resulting from the combined treatment of HT-29 with γ-IFN and 5-FU, could have also resulted in an increase of CEA mRNA available for protein synthesis.

Fig. 5A shows the results of a Northern blot analysis by using a probe, corresponding to the entire coding sequences of the CEA gene, which recognizes the BGP, NCA, and CEA transcripts. Cell treatment with γ-IFN + 5-FU resulted in increased levels of BGP (3.9, 3.7, and 1.8 kb) transcripts, with respect to treatment with the single agent. Because HT-29 cells express extremely low amounts either of CEA or NCA mRNA, hybridization with this probe does not allow a clear identification of these transcripts (32). Therefore, the same RNA samples were hybridized with a CEA-specific probe, corresponding to the 3′ untranslated region of the CEA transcript, downstream to the Alu-like sequences (27). The results (Fig. 5B) indicate an increase of the CEA transcript after exposure of the cells to 5-FU + γ-IFN, which was more evident than that detected in cells treated with 5-FU or γ-IFN alone.

Influence of 5-FU + FA on the Growth and CEA Expression of HT-29 Cells. Tumor sensitivity to 5-FU has been shown to correlate with high levels of inhibition of TS and with slow recovery of the enzyme activity. These conditions can occur only in the presence of sufficient intracellular concentrations of reduced folates. On the basis of these experimental data, we tested whether the combination FA + 5-FU could also modulate the expression of CEA. The results (Fig. 6) show that FA (69 μM) increased the antitumor effect of 5-FU at concentrations of the antimetabolite ranging from 7.8 to 125 μM. On the contrary, FA in combination with 5-FU did not modify CEA expression as determined by FACS analysis. Additional experiments showed also that higher concentrations of FA (i.e., up to 380 μM) did not influence the effect of 5-FU on CEA expression (data not shown).

Combined Effects of γ-IFN and 5-FU on HLA Class I and B7-1 Expression in HT-29 Cells. Previous studies indicated that the monomorphic component of HLA class I molecules can be up-regulated upon cell treatment with γ-IFNs of γ or α type (8, 9, 30). In this study, we have investigated whether 5-FU might affect the γ-IFN-mediated increase of HLA class I molecules. The results, obtained by FACS analysis, expressed in terms of the mean of three independent experiments (Table 1), show that: (a) treatment with 5-FU alone did not affect significantly HLA class I levels, whereas exposure to γ-IFN induced a marked increase in the expression of the same molecules (in terms of MFV only, being the percentage of HLA class I positive cells of control or of γ-IFN-treated samples, close to 100%); (b) γ-IFN + 5-FU resulted in increase of HLA class I (in terms of MFV) significantly higher than that obtainable with γ-IFN alone.

The expression of the costimulatory molecule B7-1 was also investigated, in view of its role in cell-mediated immune responses. The results (Table 1) indicate that B7-1 was not expressed in the HT-29 cell line, and that γ-IFN or 5-FU, alone or in combination, were not able to induce this antigen.

Effects of 5-FU and γ-IFN Alone or in Combination on CEA Expression in the HT-29 Tumor Xenograft. Previously, we reported that γ-IFN treatment of mice bearing human colorectal tumor xenografts increased CEA levels (15). Indeed, as summarized in Table 2, γ-IFN as well as 5-FU administration increased CEA tumor levels by 100 and 45%, respectively. Combining the agents resulted in an additive enhancement of CEA levels. Moreover, CEA was not detectable in peripheral blood of untreated tumor-bearing mice. However, detectable levels of CEA were found in the serum of animals treated with γ-IFN or 5-FU. Again, administration of the two agents resulted in CEA levels close to additive values. It is noteworthy that in the case of serum CEA, higher values of the tumor marker were detected in 5-FU-treated hosts with respect to those found in γ-IFN-treated animals.

DISCUSSION

The present report indicates that in vitro combined treatment with 5-FU and γ-IFN increases CEA expression of two colon cancer cell lines more than treatment with the single agents. Moreover, for the first time, 5-FU alone or in combina-
Drug-induced Increase of CEA Expression

An increase of the tumor marker was demonstrated at the level of cell membrane and cytoplasm and at the level of CEA gene transcription. Moreover, the pattern of mRNA transcripts revealed that 5-FU induced transcription enhancement of other molecules of the CEA family.

The results obtained in independent experiments in vitro, using graded concentrations of γ-IFN and 5-FU, did not show more than additive effects on CEA expression. However, it should be noted that combined treatment with γ-IFN + 5-FU resulted in CEA levels higher than those maximally obtainable with very high concentrations of 5-FU (i.e., more than 125 μM). This favors the hypothesis that the mechanisms responsible for CEA modulation by the two agents are different.

Little is known on the interaction between γ-IFN and 5-FU. It has been reported that γ-IFN reduces the overexpression of TS induced by 5-FU in one cell line (7). However, this was not confirmed in other colon carcinoma cell lines (6). It follows that any possible interaction between the two agents on CEA expression is presently a matter that needs to be clarified.

Experiments were carried out to explore whether one of the primary mechanisms concerning the antitumor activity of 5-FU, i.e., TS inhibition, could have any relationship with the effect of the antimetabolite on CEA expression. Therefore, the influence of FA + 5-FU on CEA expression was investigated. The reduced folate, deriving from FA, stabilizes the complex between TS and S-FU (5), thus increasing substantially the drug-mediated inactivation of this enzyme. As expected, cell treatment with FA augmented the cytotoxic activity of 5-FU (Fig. 6).

However, FA did not alter CEA modulation by 5-FU (Fig. 6). This provides indirect evidence that the FA-sensitive component of the biochemical mechanisms underlying the antitumor activity of 5-FU is distinguishable from that involved in the induction of increased CEA expression.

The enhancement of CEA protein after treatment of HT-29 cells with 5-FU + γ-IFN was more pronounced in the cytosol than in the membrane compartment with respect to cells treated with the single agents. It cannot be excluded that the increase of the protein, observed in the cytosol, might be masked at the membrane level by a marked shedding of the CEA molecule.

Increased levels of CEA protein, induced by 5-FU or γ-IFN, are paralleled by enhanced CEA gene transcription. It has been suggested that transcription factors (Sp1 and USF) could play an important role in the activation of CEA transcription mediated by γ-IFN (33). However, no data are presently available to understand the mechanism underlying the biochemical influence of 5-FU on CEA expression. Altered CEA gene regulation could be the result of increased levels of transcription factors involved in CEA expression. Actually, 5-FU treatment was found to enhance nuclear factor-kB binding activity in HIV-infected human cells in vitro (34). Moreover, the antimetabolite could produce structural changes of transcription factors that interact with the CEA gene promoter region (35). Alternatively, 5-FU could induce changes of the CEA promoter region, affecting its susceptibility to positive or negative regulation by transcription factors (35). In fact, incorporation of the fluoropyrimidine into DNA (36) and/or RNA (37) could lead to protein alteration, resulting from drug-induced miscoding (38), or errors.
of splicing of native mRNA caused by 5-FU incorporation into "small nRNA" (39-41).

The expression of cell surface antigens, including MHC class I and class II antigens, are up-regulated by γ-IFN (8, 13, 16, 42). In this case, the mechanism appears to be related to increased synthesis of guanylate-binding proteins (43, 44), which serve as mediators of signal transduction involving the Jak-Stat pathway (45). It should be pointed out that, differently from γ-IFN, 5-FU alone was not able to increase substantially the expression of histocompatibility antigens. However, the antimetabolite increased the effect of γ-IFN on HLA class I expression with a mechanism that is entirely unknown at the present time. In contrast, the two agents, alone or in combination, did not show any influence on the expression of the costimulatory molecule B7-1, in spite of previous studies in which γ-IFN was found to stimulate the expression of B7-1 molecules in monocytes (46).

The results of the in vivo studies in nude mice bearing HT-29 cells pointed out, for the first time, that 5-FU alone or in combination with γ-IFN is capable of augmenting CEA levels in tumor cells and in circulating blood (Table 1) as well. Also in this case, the antimitabolite and the cytokine showed additive effects on the expression of the tumor marker at doses that were not toxic for the host (data not shown). The presence of CEA in circulating blood could be the result of marked shedding of the protein from treated tumor cells or from dying cells, or from both. In any case, these results open up the possibility that CEA expression could be substantially augmented in tumor cells by systemic treatment with antineoplastic agents. The clinical relevance of this observation, either in terms of radioimmuno-guided surgery (47, 48) and early diagnosis of recurrence or of therapy involving CEA as target molecule (49), appears to be obvious.

In conclusion, the present report confirmed previous observations on the increase of CEA expression by 5-FU in vitro and extended these observations to a nude mouse model in vivo. When the agent was associated with one of two other drug modulators, such as γ-IFN or FA, the outcome was entirely different. In one case (i.e., γ-IFN cotreatment), the effect of the cytokine + 5-FU resulted in increment of CEA expression higher than that obtainable with each single agent, although the interaction was not more than additive. In the other case (i.e., FA cotreatment), the biomodulator increased the antitumor effect of 5-FU but did not modify its influence on CEA levels. These results provide additional information on the possible mechanism of action of 5-FU on CEA gene expression and suggest new strategies to exploit antitumor agents and biomodu-
Table 1 Effects of γ-IFN (50 IU/ml) or 5-FU (31 μM) as single agents or in combination on HLA class I and B7-1 in the HT-29 cell line

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>% PC</th>
<th>MFV</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>% PC</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99.1</td>
<td>227 ± 35</td>
<td>1.3 ± 0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU</td>
<td>99</td>
<td>275 ± 41</td>
<td>2.0 ± 0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-IFN</td>
<td>100</td>
<td>382 ± 15 &lt;0.05</td>
<td>0.9 ± 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU + γ-IFN</td>
<td>100</td>
<td>558 ± 47 &lt;0.01</td>
<td>1.1 ± 0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Same conditions used in the experiment illustrated in Fig. 1.

b % PC, percentage of positive cells; P1, probability calculated according to Student’s t-test analysis, comparing control group versus all other groups; P2, probability calculated as for P1, comparing the 5-FU-treated group versus the γ-IFN-treated group; P3, probability calculated as for P1, comparing the γ-IFN-treated group versus the group treated with 5-FU + γ-IFN; NS, not significant.

Table 2 Tumor and serum CEA levels, after treatment with 5-FU or γ-IFN, as single agents or in combination in athymic mice bearing HT-29 tumors

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Average tumor volume (mm³)</th>
<th>Serum CEA (ng/ml)</th>
<th>CEA/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>231 ± 47</td>
<td>ND</td>
<td>67.4 ± 7.7</td>
</tr>
<tr>
<td>5-FU (15 mg/kg)</td>
<td>183 ± 17b</td>
<td>18.9 ± 2.0</td>
<td>97.8 ± 12.2b</td>
</tr>
<tr>
<td>γ-IFN (10⁶ IU)</td>
<td>197 ± 30</td>
<td>6.1 ± 1.7</td>
<td>134.8 ± 14.4c</td>
</tr>
<tr>
<td>5-FU + γ-IFN</td>
<td>166 ± 21b</td>
<td>22.3 ± 4.9</td>
<td>219.0 ± 21.2b</td>
</tr>
</tbody>
</table>

a Groups of five mice were treated as described in “Materials and Methods.” ND, not detectable.

b P < 0.05 (versus average tumor volume of untreated mice).

c P < 0.05 (versus CEA levels in membranes from untreated tumors).

Acknowledgments

We thank Dr. Renato Massoud (Department of Biology, University of Rome, "Tor Vergata", Rome, Italy) for quantitation of Western blot analysis by densitometer.

References


**Clinical Cancer Research**

**Effect of the combined treatment with 5-fluorouracil, gamma-interferon or folinic acid on carcinoembryonic antigen expression in colon cancer cells.**

A Aquino, S P Prete, J W Greiner, et al.


| Updated version | Access the most recent version of this article at: [http://clincancerres.aacrjournals.org/content/4/10/2473](http://clincancerres.aacrjournals.org/content/4/10/2473) |

| E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |
| Reprints and Subscriptions | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| Permissions | To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org. |