5-Fluorouracil Suppresses Nitric Oxide Biosynthesis in Colon Carcinoma Cells

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Abstract

Nitric oxide is an important cellular mediator that plays a role in regulating cellular proliferation of both normal and tumor cells. In the present study, we characterized nitric oxide production by the human colon adenocarcinoma cell line DLD-1 and examined the effects of 5-fluorouracil (5-FUra), an antimetabolite effective against colon tumors, on nitric oxide production. IFN-γ was found to be a potent inducer of nitric oxide production in DLD-1 cells. This effect was dependent on l-arginine and blocked by the nitric oxide synthase inhibitors N\textsuperscript{\textprime}L-monomethyl-L-arginine, nitroarginine, and aminoguanidine. Production of nitric oxide by DLD-1 cells was due to the expression of the inducible (type II) form of nitric oxide synthase. mRNA for the nitric oxide synthase was present in both untreated and IFN-γ-stimulated cells, as determined by RT-PCR, suggesting that expression of enzyme is regulated posttranscriptionally. Treatment of DLD-1 cells with concentrations of 5-FUra that are not growth inhibitory or cytotoxic strongly inhibited their ability to express nitric oxide synthase and produce nitric oxide in response to IFN-γ. This effect was not reversed with thymidine, indicating that inhibition of nitric oxide production was due to incorporation of 5-FUra into RNA. However, pretreatment of DLD-1 cells with 5-FUra before stimulation with IFN-γ also suppressed nitric oxide production. Thus, inhibition of nitric oxide production was not due directly to incorporation of 5-FUra into the mRNA for nitric oxide synthase. Taken together, these data suggest that inhibition of nitric oxide biosynthesis in colon tumor cells by 5-FUra may underlie, at least in part, the efficacy of this antitumor agent.

Introduction

Nitric oxide is an important vasoactive mediator that plays a key role in regulating tumor growth, invasion, and angiogenesis (1). It is produced by many tumor cells after stimulation with cytokines such as IFN-γ (2, 3). To initiate its biological activity, nitric oxide activates critical components of a variety of cellular signal transduction pathways, including those responsible for controlling vascular tone such as guanylyl cyclase, cGMP-dependent protein kinases, and calcium-dependent membrane potassium channels (4). It is now well recognized that effective strategies for limiting tumor growth include not only inhibiting DNA replication but also interfering with the ability of growth factors and cytokines to regulate processes required for proliferation and survival, such as neovascularization. Because nitric oxide appears to be involved in many of these processes, interfering with its production in tumors may be an important target for chemotherapy. The antimetabolite 5-FUra\textsuperscript{2} is one of the more prominent clinical antitumor agents and one of the few drugs that displays significant activity toward colorectal cancer (5). Although it has been almost 40 years since the discovery of 5-FUra, the precise mechanism by which it inhibits tumor growth is not known. Metabolic conversion to 5-fluorouridine 5'-triphosphate with subsequent incorporation into RNA and/or the formation of 5-fluorodeoxyuridyl acid, a well-recognized inhibitor of thymidylate synthetase, are thought to contribute to its actions (6). Small amounts of 5-FUra also incorporate into DNA (6). In the present study, we report that in a process independent of thymidylate synthetase inhibition, 5-FUra suppresses cytokine-stimulated nitric oxide production by colon carcinoma cells. Given the potentially important functions of nitric oxide in regulating tumor development, our observations suggest a novel mechanism underlying the effectiveness of 5-FUra against colon cancer.

Materials and Methods

Chemicals and Supplies. Tissue culture medium and all other cell culture supplies were obtained from Life Technologies, Inc./BRL (Gaithersburg, MD). \textsuperscript{[3H]}Arginine (specific activity, 57 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE). Unlabeled and labeled recombinant human IFN-γ was kindly provided by Sidney Pestka. University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School (Piscataway, NJ). Disuccinimyl suberate was obtained from Pierce Chemical Co. (Rockford, IL). NMMA and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Cultures. DLD-1 colon carcinoma cells, obtained from the American Type Culture Collection, were grown in monolayer culture in DMEM supplemented with 10% FCS. Nitric oxide production was measured in cells grown in 24-well tissue culture dishes as described previously (7). Before initiating experiments, cells (approximately 50–60% confluent) were washed twice with PBS and refed with serum- and phenol red-free DMEM. Cells were stimulated to produce nitric oxide by the addition of human recombinant IFN-γ to the culture medium. Nitric oxide production by the cells was quantified by measuring the accumulation of nitrate in the culture medium using the Griess reaction with sodium nitrite as the standard (7). To measure the effects of the nitric oxide synthase inhibitors on nitric oxide production, cells were treated with the compounds and 100 units/ml IFN-γ for 48 h.

Nitric Oxide Synthase in DLD-1 Cells. Nitric oxide synthase activity was measured by the metabolic conversion of \textsuperscript{[3H]}arginine to \textsuperscript{[3H]}citrulline (4). DLD-1 cells grown to 50–60% confluence in 6-well culture dishes were stimulated to produce nitric oxide with 100 units/ml IFN-γ for 48 h and then incubated with 1 mL of \textsuperscript{[3H]}arginine (5 μCi/mL, 10 μM final concentration) in HBSS supplemented with 20 mM HEPES (pH 7.4) at 37°C. After appropriate time intervals, the labeling medium was removed, the cells were rinsed with ice-cold PBS (3 × 5 ml), and 1 ml of ice-cold 5% trichloroacetic acid was added to precipitate proteins and extract cystosolic amino acids. After 15 min, aliquots of the acid-soluble fraction were counted for radioactivity or analyzed by HPLC with radiometric detection using a Whatman JQ SCX (5-μm particle size, 4.6 × 250 mm) cation exchange column. The samples were eluted at a flow rate of 1 ml/min by a step gradient consisting of 0–6 min, 100% buffer A (190 mM citric acid monohydrate-10 mM sodium citrate) and 0% buffer B (0.2 M sodium citrate); 6–40 min, a linear gradient to 50% buffer B; and 40–50 min, 100% buffer B. Peaks were detected with a Packard Radiomatic model A110 on-line scintillation counter equipped with a 500-μl flow cell using a flow rate of 3 ml/min for the scintillation fluid (Monoflow 5, National Diagnostics, Atlanta, GA). Under these conditions, the retention times for citrulline, ornithine, and arginine were 12.4, 37.7, and 43.3 min, respectively. Precipitated proteins on the cultures plates were washed (3 × 5 ml) with ice-cold 5% trichloroacetic acid, solubilized with 1 ml 0.2 N NaOH, and counted for radioactivity.

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3 The abbreviations used are: 5-FUra, 5-fluorouracil; NMMA, N\textsuperscript{\textprime}L-monomethyl-L-arginine.

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For RT-PCR of nitric oxide synthase, RNA was isolated from control and treated DLD-1 cells by guanidinium thiocyanate/phenol/chloroform extraction using the TRizol reagent (Life Technologies, Inc.). A total of 25 ng of template RNA and 100 ng of each nucleotide primer [5'-TCCATOCAGAACCTT-3' (bases 1425-1441, sense) and 5'-TCCATOCAGAACCTT-3' (bases 1908-1924, antisense); Ref. 2] were used in the reactions. Denaturation, annealing, and elongation temperatures for RT-PCR were 96°C (35 s), 56°C (2 min), and 72°C (2 min), respectively. Only a single RT-PCR product (499 bp) was obtained from the cells.

For Western blotting of nitric oxide synthase, control and treated DLD-1 cells first lysed in buffer consisting of 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 10 mM Tris-HCl, pH 7.4. Cell extracts were then fractionated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed with monoclonal antibodies to the inducible (Type II) form of nitric oxide synthase (Transduction Laboratories, Lexington, KY) using an alkaline phosphatase-conjugated secondary antibody and a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate detection system (Bio-Rad, Hercules, CA). Each lane on the gel contained 25 μg of protein.

Receptor Binding and Covalent Cross-Linking of IFN-γ. Receptor binding assays for IFN-γ and covalent receptor cross-linking experiments were performed as described by Mariano and Pestka (8). Scatchard analysis was used to determine the Kd and the number of receptors per cell. For covalent cross-linking of [32P]-IFN-γ-receptor complexes on DLD-1 cells, 0.5 X 10⁶ cells in 0.3 ml were incubated with 100,000 cpm of [32P]-IFN-γ (specific activity, 119 μCi/μg) with or without 1 μg of unlabeled IFN-γ. After 90 min at room temperature, 0.5 mM disuccinimidyl suberate was added. After an additional 20 min on ice, the reaction was quenched by the addition of Tris-HCl (pH 7.5; 20 mM final concentration). Cells were pelleted, extracted with 120 μl PBS containing 0.5% (v/v) Triton X-100 and 5 mM EDTA, and centrifuged (15,000 g for 10 min at 4°C). Supernatants were separated on 7.5% SDS-polyacrylamide gels and the [32P]-IFN-γ-receptor complex detected by autoradiography.

Results and Discussion

In initial experiments, we characterized nitric oxide production by DLD-1 colon carcinoma cells. We found that treatment of the cells with IFN-γ readily stimulated production of nitric oxide (Fig. 1). This required l-arginine and was concentration- and time-dependent (Fig. 1 and not shown). In mammalian cells, nitric oxide is generated in cells by the NADPH-dependent oxidation of arginine to citrulline by the enzyme nitric oxide synthase (4). Three specific inhibitors of nitric oxide synthase, NMMA, nitroarginine, and aminoguanidine, were found to inhibit nitric oxide production by the DLD-1 cells (Fig. 1).

We next assayed nitric oxide synthase activity in DLD-1 cells by incubating the cultures with [3H]arginine and then measuring accumulation of [3H]citrulline. Under our assay conditions, [3H]arginine was readily taken up by DLD-1 cells. Approximately 90% of the label accumulated in acid-soluble cytosolic pools of the cells, and 10% was incorporated into protein (not shown). HPLC analysis of acid-soluble pools from IFN-γ-treated cells revealed that approximately 4-5% of the [3H]arginine was converted to [3H]citrulline (Fig. 2), a reaction that was inhibited by NMMA (not shown). Interestingly, approximately 10% of the label was also metabolized to [3H]ornithine, indicating that the cells contained arginase activity (not shown). In further studies using Western blotting, we found that IFN-γ-stimulated expression of protein for the inducible form of nitric oxide synthase in DLD-1 cells (Fig. 3). Using RT-PCR, mRNA for this isoform of the enzyme was identified in both untreated and IFN-γ-stimulated cells (Fig. 3). Constitutive expression of mRNA for inducible nitric oxide synthase in DLD-1 cells has previously been reported (2). These data suggest that nitric oxide synthase expression in these cells may be regulated posttranscriptionally. In this regard, alternative splicing of the mRNA for inducible nitric oxide synthase has been demonstrated in DLD-1 cells (9) and this may be important in regulating expression of nitric oxide synthase protein. It should be noted that it appeared that more RT-PCR product for inducible nitric oxide synthase was evident in IFN-γ-treated cells. However, we found that this varied between experiments, possibly because of the fact that this technique is not quantitative.

We next examined the effects of 5-FUra on nitric oxide production in DLD-1 cells stimulated with IFN-γ. 5-FUra, at concentrations that did not prevent the cells from reaching confluence (3 X 10⁻⁷ to 3 X 10⁻⁵ M), was found to suppress the ability of IFN-γ to induce production of nitric oxide (Fig. 4 and not shown). 5-FUra alone had no effect on nitric oxide production by the cells. At the concentrations tested, no cytotoxicity was observed, as determined by trypan blue dye exclusion and by the fact that the cells continued to grow. In further experiments, we found that both continuous and simultaneous exposure to 5-FUra was not required for this inhibition because preincubation of the cells with the drug (3 X 10⁻⁵ M) for as little as 3 h also suppressed subsequent IFN-γ-stimulated nitric oxide production (Fig. 4). Inhibition of nitric oxide production in IFN-γ-treated DLD-1 cells by 5-FUra was due to suppression of nitric oxide synthase activity. Thus, we found a 20-fold decline in [3H]citrulline production 48 h after treatment with IFN-γ; B, time course of nitrite accumulation; C, effects of nitric oxide synthase inhibitors on nitric oxide production. Cells were treated with the inhibitors and 100 units/ml IFN-γ for 48 h.

![Graphs](https://example.com/graphs.png)
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Fig. 2. Nitric oxide synthase activity in DLD-1 cells. Nitric oxide synthase activity in DLD-1 cells treated with 100 units/ml IFN-γ for 48 h or IFN-γ plus 5-FUra (3 × 10⁻⁵ M) was assayed by measuring the ability of the cells to convert [³H]arginine to [³H]citrulline as described in "Materials and Methods." Bars, SE.

formed (to less than 0.6% of total acid soluble radioactivity) from [³H]arginine after 5-FUra-treatment (Fig. 2). Under these conditions, 5-FUra had no effect on the uptake of [³H]arginine either into acid-soluble cytoplasmic pools or acid-insoluble proteins in DLD-1 cells (not shown). We also observed a 50% depression in the formation of [³H]ornithine in the cells, which may be due to decreased arginase activity. In this regard, arginase activity is known to be highly sensitivity to nutrient supply and growth conditions (10). Western blot analysis of the cells revealed that treatment with 5-FUra suppressed the ability of the cells to produce inducible nitric oxide synthase protein in response to IFN-γ (Fig. 3). Inducible nitric oxide synthase mRNA, as determined by RT-PCR, was present in both control and 5-FUra-treated cells (Fig. 3). Taken together, these data indicate that there is a 5-FUra-sensitive step, possibly in the final processing of the mRNA for inducible nitric oxide synthase and/or in translation into protein, that prevents expression of enzyme activity in the cells.

A question arises as to the mechanism by which 5-FUra suppresses inducible nitric oxide synthase expression in DLD-1 cells. If 5-FUra inhibits nitric oxide production via blockade of thymidylate synthase, then it would be expected that thymidine, which overcomes this inhibition by providing thymidine nucleotides by way of salvage pathways, would prevent this effect. However, we found that thymidine was unable to overcome the inhibitory effects of 5-FUra on nitric oxide production (Fig. 4). These data indicate that RNA is the likely target for this drug in DLD-1 cells. In this regard, we have previously demonstrated that the sensitivity of many human tumor cells to 5-FUra is dependent on its incorporation into RNA (11). At the present time, the type of RNA altered by 5-FUra in DLD-1 cells that prevents expression of nitric oxide synthase protein is not known. There are many different types of RNA molecules, the functions of which may be sensitive to 5-FUra substitution. Selectivity of 5-FUra toward any particular type of RNA may depend upon its function, the extent to which it is incorporated into the nucleic acid, and its persistence in the cell, each of which may be regulated by rates of synthesis and/or degradation of the RNA. In some systems, 5-FUra has been reported to inhibit maturation of rRNA (12) as well as mRNA (13). This latter effect may be due to inhibition of pre-mRNA splicing (13). 5-FUra-substituted snRNAs are known to inhibit mRNA precursor splicing, possibly by altering the recognition and binding of critical proteins in the spliceosome complex (13), and this may occur.

Fig. 3. Expression of nitric oxide synthase and the IFN-γ receptor in DLD-1 cells. A. immunoblot analysis of inducible nitric oxide synthase. Cells were preincubated in medium with and without 3 × 10⁻⁵ M 5-FUra for 24 h. They were then rinsed free of the drug and incubated with medium in the presence or absence of 100 units/ml IFN-γ. After 48 h, cell lysates were prepared and analyzed for inducible nitric oxide synthase by Western blotting as described in "Materials and Methods." Lane 1, control cells; Lane 2, cells treated with IFN-γ; Lane 3, cells treated with 5-FUra; Lane 4, cells treated with 5-FUra and IFN-γ. B. RT-PCR analysis of inducible nitric oxide synthase. RNA was isolated from the cells, and RT-PCR was performed as indicated in "Materials and Methods." Lane 1, control cells; Lane 2, cells treated with IFN-γ; Lane 3, cells treated with 5-FUra; Lane 4, cells treated with 5-FUra and IFN-γ. C. Covalent cross-linking of [³P]-labeled IFN-γ to DLD-1 cells. [³P]-labeled IFN-γ receptor cross-linking in DLD-1 cells was performed as indicated in "Materials and Methods." Autoradiography was used to visualize the [³P]-IFN-γ-receptor complex. Lane 1, cellular extracts from control cells; Lane 2, cellular extracts from cells treated with 3 × 10⁻⁵ M 5-FUra for 24 h; Lane 3, cellular extracts from 5-FUra-treated cells labeled with [³P]-IFN-γ in the presence of an excess (1 µg/ml) of unlabeled IFN-γ; IFN-R, [³P]-IFN-γ-receptor complex.
concentrations of 5-FUra for 12 h. IFN-γ was added during the final 48 h of incubation to stimulate nitric oxide production. B. effects of preincubating DLD-1 cells with 5-FUra on subsequent IFN-γ-stimulated production of nitric oxide. Cells were treated with 3 × 10^{-5} M 5-FUra for the indicated periods of time, rinsed free of the drug, and then stimulated with 100 units/ml IFN-γ. Nitric oxide production by the cells was quantified 48 h later. C. lack of an effect of thymidine on the ability of 5-FUra to inhibit IFN-γ-stimulated nitric oxide production. Cells were treated with 3 × 10^{-2} M 5-FUra and/or 3 × 10^{-5} M thymidine for 12 h and then rinsed free of the drugs. After an additional 12 h, the cells were treated with 100 units/ml IFN-γ. Nitric oxide production by the cells was quantified 48 h later. Bars: SE.

in 5-FUra treated DLD-1 cells. In the present studies, because pre-treatment of DLD-1 cells with 5-FUra for as little as 3 h also suppressed subsequent IFN-γ-stimulated nitric oxide production, its effects are probably not due to direct incorporation into mRNA for inducible nitric oxide synthase protein. One could speculate that 5-FUra is incorporated into a relatively long-lived RNA species important in the final stages of processing or translation of the mRNA for inducible nitric oxide synthase.

At the present time, we cannot rule out the possibility that 5-FUra acts by disrupting the IFN-γ signaling pathway in DLD-1 cells. This could occur if 5-FUra inhibited expression of receptors for IFN-γ on DLD-1 cells. To test this possibility, we examined IFN-γ receptor binding using 32P-labeled IFN-γ as the radioligand (8). Scatchard analysis (Ref. 8 and not shown) revealed that the cells contained high-affinity receptors (Kd = 7.5 × 10^{-10} M, with 1.1 × 10^4 receptors/cell) on the cells that were not significantly altered after 5-FUra treatment (3 × 10^{-2} M for 24 h; Kd = 9 × 10^{-10} M, with 1.2 × 10^4 receptors/cell). Cross-linking of the receptor with the 32P-labeled IFN-γ followed by electrophoresis on SDS-polyacrylamide gels revealed a molecular weight of 110,000 for the 32P-IFN-γ-receptor complex (Fig. 3). Cross-linking of the receptor was prevented by unlabeled IFN-γ demonstrating specificity of the binding. Neither the molecular weight nor the extent of receptor cross-linking was altered in the cells after 5-FUra treatment (Fig. 3). IFN-γ receptor binding is known to lead to activation of cytosolic tyrosine kinases that initiate nuclear translocation and activation of the signal transduction and activation of transcription class of transcription factors (14). Activated transcription complexes have the capacity to bind to promoter regions of a spectrum of IFN-γ-stimulated genes, including inducible nitric oxide synthase (9). Further studies are required to determine whether 5-FUra-treatment of DLD-1 cells modulates the expression and activity of these important transcription factors.

Many colon cancer cells express nitric oxide synthase activity (2, 3). Because growth, invasion, and metastases of colon tumors are enhanced by expression of nitric oxide synthase activity (1), it is possible that disrupting the production of this reactive nitrogen intermediate may be an important route toward inhibiting development of this neoplasm. Thus, our findings that 5-FUra effectively suppresses induction of nitric oxide biosynthesis in colon tumor cells may be particularly relevant and explain, at least in part, the efficacy of this antitumor agent toward colorectal cancer. It should be noted that the ability of cancer cells to produce nitric oxide is not always directly correlated with tumor progression. For example, nitric oxide synthase activity, as measured by the reduction of nitroblue tetrazolium, has been reported to be inversely correlated with the progression of some human colon tumors (15). However, these experiments must be interpreted with caution because the four-electron reduction of nitroblue tetrazolium can also be initiated by a variety of free radicals besides nitric oxide, including superoxide, hydroxyl radical, and organic free radicals (16). Nitric oxide can be cytotoxic for tumor cells (17), and limited metastases of melanoma cells, as well as regression of reticulum cell sarcoma metastases, have been correlated with expression of inducible nitric oxide synthase (18, 19). Xie et al. (20) also report that transfection of murine melanoma cells with the inducible nitric oxide synthase gene suppresses tumorigenicity and metastasis, possibly by inducing apoptosis in the tumor cells. It may be that the role of nitric oxide in tumor development is dependent on the cell type and/or the role of apoptosis in tumor development.

Our studies show that 5-FUra has the capacity to suppress nitric oxide production in DLD-1 cells in culture in the absence of toxicity. Nitric oxide is a potent vasodilating agent (2), and in well-vascularized tumors, 5-FUra-induced inhibition of nitric oxide production may result in decreased nutrient supply due to reduced blood flow. Nitric oxide has also been reported to enhance neovascularization (1), and its inhibition by 5-FUra would also slow tumor growth and possibly cause tumor regression. It is well documented that in tumors, 5-FUra acts directly to inhibit cell growth and viability. Thus, 5-FUra may be effective as an antitumor agent because it has multiple sites of action. Limiting cell growth in tumors may also allow a more effective immunological challenge to cause tumor regression. Further studies are needed to explore the role of nitric oxide in regulating the growth of colon cancer cells.

References

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