Folic acid-mediated inhibition of serum-induced activation of EGFR promoter in colon cancer cells

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Nagothu, Kiran K., Arun K. Rishi, Richard Jaszewski, Omer Kucuk, and Adhip P. N. Majumdar. Folic acid-mediated inhibition of serum-induced activation of EGFR promoter in colon cancer cells. Am J Physiol Gastrointest Liver Physiol 287: G541–G546, 2004. First published April 8, 2004; 10.1152/ajpgi.00365.2003.—Although accumulating evidence suggests a chemopreventive role for folic acid (FA) in colorectal carcinogenesis, the underlying mechanisms are largely unknown. Previously, we reported that supplemental FA inhibits the expression and activation of epidermal growth factor receptor (EGFR) in colon cancer cell lines. To determine the mechanism(s) by which FA affects EGFR function, we have examined whether and to what extent supplemental FA or its metabolites 5-methyltetrahydrofolate (5-MTF), dihydrofolate (DF), and tetrahydrofolate (TF) will modulate basal and serum-induced activation of the EGFR promoter in the HCT-116 colon cancer cell line. HCT-116 cells were preincubated with or without (control) FA or one of its metabolites (10 μg/ml) for 48 h, transfected with the EGFR promoter luciferase reporter construct, and incubated for 48 h with FA, DF, TF, or 5-MTF in the absence or presence of 10% FBS. Supplemental FA as well as its metabolites markedly inhibited EGFR promoter activity and its methylation status. Exposure of the cells to 10% FBS caused a marked stimulation of EGFR promoter activity and its expression, both of which were greatly abrogated by supplemental FA and 5-MTF. In contrast, serum-induced activation of c-fos promoter activity was unaffected by 5-MTF. The 5-MTF-induced inhibition of serum-mediated stimulation of EGFR promoter activity and EGFR expression was reversed when methylation was inhibited by 5-aza-2’-deoxycytidine. Our data suggest that FA and its metabolite 5-MTF inhibit EGFR promoter activity in colon cancer cells by enhancing methylation. This could partly be responsible for FA-mediated inhibition of growth-related processes in colorectal neoplasia.

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colorectal carcinogenesis; methylation; 5-methyltetrahydrofolate

ACCUMULATING EVIDENCE from epidemiological studies, human biopsies, and animal and cell culture studies suggests a chemopreventive role for folic acid (FA) in colorectal carcinogenesis (6, 7, 22, 28). Results from these studies have demonstrated that a diet deficient in FA is associated with an increased risk of colorectal neoplasia (2, 5), whereas dietary supplementation of this nutrient is chemopreventive (14, 15). Meenan et al. (18) measured FA levels in adenoma, carcinoma, and normal-appearing adjacent mucosa and found that the levels were lower in adenoma and carcinoma than in normal-appearing mucosa. This suggests that mucosal FA may play a role in the development of colorectal neoplasia. We demonstrated that, in rats, supplemental FA suppresses carcinogen-induced colonic mucosal hyperproliferation as reflected by ornithine decarboxylase and tyrosine kinase activities (20). We further demonstrated that supplemental FA markedly inhibits proliferation of the colon cancer cell lines HCT-116 and Caco-2 (13). A similar observation was also made by Akoglu et al. (1), who noted that DNA synthesis in Caco-2 cells was inhibited not only by supplemental FA, but also by its metabolite 5-methyltetrahydrofolate (5-MTF).

The regulatory mechanisms utilized by FA in exerting its chemopreventive effect(s) on colorectal neoplasia are poorly understood. However, we observed that the FA-dependent inhibition of proliferation of colon cancer cell lines is accompanied by a concomitant reduction in expression and tyrosine kinase activity of epidermal growth factor receptor (EGFR) (13). Because EGFR, a 170-kDa transmembrane glycoprotein, plays a critical role in regulating the development and progression of colorectal cancer (8), we hypothesized that FA may exert its chemopreventive role by modulating the EGFR signaling processes. Therefore, to determine whether supplemental FA will affect the functional properties of EGFR, we examined whether and to what extent FA or its metabolite(s) will modulate serum-induced changes in EGFR promoter activation in colon cancer cells.

FA is the primary methyl donor for DNA methylation and for producing the purines and pyrimidines required for DNA synthesis. Lack of FA or methyl group nutrients in the diet has been shown to cause DNA hypomethylation in rats and humans, whereas an opposite phenomenon is noted with excess FA (10, 11, 24). Gene hypermethylation, particularly within the promoter, has been shown to cause tissue-specific gene silencing. It has been demonstrated that methylation of CpG sequences within promoters can inhibit binding of transcription factors, which is thought to be one of the mechanisms for gene inactivation in neoplastic cells and tissue-specific gene expression (3, 17, 21, 26, 28). In view of this, we further examined the mechanism(s) underlying FA-mediated inhibition of EGFR activation. Here, we report that FA, as well as its metabolite 5-MTF, specifically inhibits basal and serum-induced activation of the EGFR promoter in HCT-116 colon cancer cells that could partly be attributed to alterations in methylation.

METHODS

Materials. The human colon cancer cell line HCT-116 (American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and...
M 5-aza-2-deoxycytidine, a known DNA methylation inhibitor. /H11032 luciferase assay. In some experiments, the cells were treated with 1 harvest 48 h after transfection, and extracts were prepared for normalize for differences in transfection. Afte r3ho ftransfection, the Escherichia coli lacZ manufacturer constructs by Lipofectamine (Invitrogen) essentially according to the /H9252 concentration by a protein assay kit (Bio-Rad).

**RESULTS**

The first set of experiments was undertaken to determine time-course effects of EGFR promoter activation in HCT-116 cells in response to 10% FBS. In this and subsequent investigations, rat EGFR promoter constructs were utilized. Although there is ~20% sequence homology between human and rat EGFR promoters (16), the fact that rat promoter constructs have been successfully utilized to study EGFR regulation in different mammalian cell lines suggests a conservation of critical promoter-dependent pathways of EGFR regulation (16). For this reason, we utilized these constructs to study their regulation in human colon cancer cell lines. After transfection with the EGFR promoter luciferase reporter construct pRE1102-Luc, HCT-116 cells were incubated

**Methylation-specific PCR analysis of human EGFR promoter.** HCT-116 colon cancer cells were incubated in the absence (control) or presence of 5-MTF (10 µg/ml) for 48 h. Genomic DNA was extracted as described previously (19). Bisulphite modification of DNA to convert all unmethylated cytosines to uracil and then to thymidine during the subsequent PCR step while leaving the methylated cytosines unaffected was performed as described by Herman et al. (9).

**Fig. 1.** Schematic representation of different epidermal growth factor receptor (EGFR) promoter luciferase constructs: pRE260 contains 260 nt of EGFR promoter, pRE1102 contains 1,102 nt of EGFR promoter, and the reporter construct pRE1102A260 lacks 260 nt of the pRE1102 construct. Putative locations of TCC repeat, Sp1 motif, and methylation sites are indicated. 1% antibiotic-antimycotic ( Gibco-BRL, Bethesda, MD). Plasmid constructs, pRE1102-Luc (−1102 to −2), pRE260, and pRE1102A260, that contain fragments of the rat EGFR promoter-driving luciferase reporter gene (15) were obtained from Dr. Johnson (National Institutes of Health, Bethesda, MD). Figure 1 provides a schematic representation of different EGFR promoter constructs. 

**Cell culture.** Cells (~1 × 10^6) were plated in duplicate in six-well plates and incubated for 48 h in the absence (control) or presence of FA, DF, or 5-MTF, each at 10 µg/ml. The cells were serum starved for 24 h and then transfected with one of the EGFR or c-fos promoter-driving constructs by Lipofectamine (Invitrogen) essentially according to the manufacturer’s instructions. The plasmid pRSVZ, which carries the Escherichia coli lacZ gene under the control of the Rous sarcoma long terminal repeat and encodes for β-galactosidase, was cotransfected to normalize for differences in transfection. After 3 h of transfection, the cells were incubated with fresh medium ( RPMI) containing FA or its metabolites dihydrofolate (DF), tetrahydrofolate (TF), and 5-MTF, and 5-aza-2'-deoxycytidine were purchased from Sigma (St. Louis, MO). EGFR antibodies (rabbit polyclonal) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Western blot analysis.** Cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 2.5 mM NaVO₃, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml each aprotinin and leupeptin, and 50 µg/ml soybean trypsin inhibitor). The lysates were rotated for 30 min at 4°C and subsequently centrifuged at 11,000 g for 15 min at 4°C. The supernatant was used for Western blot analysis after determination of protein concentration by a protein assay kit (Bio-Rad).

Aliquots containing 50 µg of protein were separated on a 7.5% SDS-polyacrylamide gel and then electroblotted to a nitrocellulose membrane. The membrane was blocked overnight with 5% nonfat dry milk in TBST buffer (20 mM Tris, pH 7.6, 100 mM NaCl, and 0.1% Tween 20) and then incubated for 3 h with the primary antibody (1:1,000 dilution) in TBST buffer containing 5% nonfat dry milk at room temperature. After they were washed three times with TBST buffer, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000 dilution) for 1 h at room temperature. Proteins were visualized using enzyme-linked enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL). The membranes were stripped and probed with β-actin antibodies (Boehringer-Mannheim) as an internal control. Signals on the blots were visualized by autoradiography and quantitated by densitometry using the ImageQuant image analysis system (Storm Optical Scanner, Molecular Dynamics, Sunnyvale, CA).

**Reporter gene assay.** Cells were transfected with 0.8 µg of the noted plasmid and 0.2 µg of internal control pRSVZ plasmid. After the treatment, the cells were lysed in lysis buffer (25 mM glycyglycine, 15 mM MgSO₄, 4 mM EGTA, 1% Triton X-100, and 0.1 mM DTT) and then clarified by centrifugation at 10,000 g for 5 min. The supernatant was used for luciferase assay. Luciferase activities were measured in a luminometer (AutoLumat Plus, Berthold Tech). Transfection efficiency was normalized to β-galactosidase activity. Values are means ± SE.
in the absence (control) or presence of 10% FBS for 24, 48, or 72 h. In the presence of serum, the activity of pRE1102-Luc was increased by 50, 105, and 102% at 24, 48, and 72 h, respectively, compared with the corresponding controls (Fig. 2). The 48-h time point was chosen for all subsequent experiments.

To determine whether and to what extent FA or its metabolites will affect the serum-induced activation of EGFR promoter constructs, we conducted the next set of experiments. HCT-116 cells were incubated in the absence (control) or presence of FA, DF, TF, or 5-MTF, each at 10 μg/ml, for 48 h, transfected with pRE1102-Luc, and then incubated in the absence or presence of 10% FBS. These concentrations were chosen, because they were previously shown to inhibit proliferation of colon cancer cells (1). In the absence of serum, supplemental FA and each of its metabolites caused a marked inhibition of EGFR promoter activation (Fig. 3). 5-MTF was most effective in this regard, revealing a 75% inhibition compared with the controls (Fig. 3). However, in the absence of FA or its metabolites, serum caused a marked activation of the EGFR promoter, revealing a 75–250% stimulation over the controls (Fig. 3). This stimulation was attenuated by 50–70% when the cells were preincubated with supplemental FA or 5-MTF (Fig. 3). Neither DF nor TF was effective in this regard (Fig. 3).

To determine the EGFR promoter sequences involved in MTF-dependent regulation, additional EGFR luciferase reporter constructs pRE1102A260 and pRE260 (Fig. 1) were utilized. In this and subsequent experiments, 5-MTF was used, because it was found to be most effective in suppressing the serum-induced activation of the EGFR promoter construct pRE1102. HCT-116 cells were transfected with promoter constructs and subsequently incubated for 48 h in the absence or presence of 10% FBS and/or 5-MTF. Serum-induced activation of all EGFR promoter constructs was modulated by 5-MTF (Fig. 4). As has been observed for pRE1102-Luc, in the absence of supplemental 5-MTF, activity of pRE1102-Luc, pRE1102Δ260-Luc, and pRE260-Luc constructs was increased by 75, 250, and 50%, respectively, in response to serum compared with the corresponding controls (Fig. 4). However, the serum-induced activation of each of the EGFR promoter constructs was greatly attenuated by supplemental 5-MTF (Fig. 4).

In contrast to what has been observed for the EGFR promoter(s), preincubation of HCT-116 cells with supplemental 5-MTF produced no inhibition of the serum-induced activation of the c-fos promoter (Fig. 5). A small 30% increase in c-fos promoter activity was observed when HCT-116 cells were exposed to FBS and 5-MTF compared with the level noted with FBS alone (Fig. 5). The lack of FA/MTF-dependent inhibition of c-fos promoter activity suggests that the effect of FA is specific to EGFR regulation.

To further determine whether the observed changes in EGFR promoter activation in response to serum and/or 5-MTF are reflected in EGFR expression, the levels of EGFR were analyzed after transfection of HCT-116 cells with the EGFR promoter construct pRE1102-Luc and subsequent exposure to 10% FBS in the presence or absence of 5-MTF as described above. In the absence of serum, 5-MTF caused a minor (10–20%) reduction in EGFR levels compared with controls (Fig. 6). On the other hand, in the absence of 5-MTF, serum caused a ~50% increase in EGFR levels compared with cells that were not incubated with serum or 5-MTF (Fig. 6). How-

![Fig. 2](http://ajpgi.physiology.org/)

Fig. 2. Time-course changes in activation of EGFR promoter pRE1102 in the presence of 10% FBS compared with corresponding values in the absence of FBS. Values are means ± SE of 4 independent experiments. *P < 0.01 vs. corresponding control.

![Fig. 3](http://ajpgi.physiology.org/)

Fig. 3. Effects of supplemental folic acid (FA), dihydrofolate (DF), tetrahydrofolate (TF), or 5-methyltetrahydrofolate (5-MTF) on constitutive and serum-induced activation of EGFR promoter pRE1102. *P < 0.01 vs. corresponding control (i.e., without FBS and supplemental FA, DF, or 5-MTF). †P < 0.01 vs. serum-induced levels. Values are means ± SE of 4 experiments.
ever, this stimulation was totally abrogated by supplemental 5-MTF (Fig. 6).

Methylation of CpG sequences within promoters is known to inhibit binding of transcription factors and, thereby, suppress gene activation (20–24). Because FA is the primary donor of DNA methylation, we examined whether 5-MTF-mediated inhibition of serum-induced activation of the EGFR promoter, as well as levels of the receptor, could partly be attributed to hypermethylation of the EGFR promoter. To test this possibility, 5-aza-2′-deoxycytidine, an inhibitor of DNA methylation, was added to cells that were incubated with 5-MTF, transfected with the EGFR promoter construct, and then incubated again with 5-MTF in the absence or presence of 10% FBS. As has been observed earlier, serum, in the absence of 5-MTF, caused ~40% activation of the EGFR promoter luciferase construct pRE1102-Luc, which was greatly abrogated by 5-MTF (Fig. 7). However, in the presence of 5-aza-2′-deoxycytidine, 5-MTF-induced inhibition of EGFR promoter activation by serum was completely reversed (Fig. 7). These changes were reflected in EGFR expression, as evidenced by alterations in EGFR levels (Fig. 8).

To further ascertain whether 5-MTF treatments result in methylation of the EGFR promoter, the following experiments were conducted. HCT-116 cells were incubated in the absence (control) or presence of 5-MTF (10 μg/ml) for 24 h. Genomic DNAs from untreated (controls) and 5-MTF-treated HCT-116 cells were subjected to bisulfite modification followed by methylation-specific PCR. When primers for amplification of unmethylated DNA are used, it is anticipated that higher levels of promoter DNA amplification will occur in controls than in their MTF-treated counterparts. On the other hand, utilization of primers for methylated DNA will result in increased amplification of the promoter DNA in MTF-treated cells. Indeed, our results show increased EGFR promoter amplification in controls with primers for unmethylated DNA compared with the DNA from MTF-treated cells (Fig. 9, lane 1 vs. lane 2). In contrast, increased promoter amplification occurred with primers for methylated DNA from MTF-treated cells (Fig. 9, lane 3 vs. lane 4).

**DISCUSSION**

Although the epidemiology of colorectal cancer is clearly related to genetic susceptibility, dietary factors such as vit...
Folic acid and EGFR promoter activation

Mins and micronutrients are thought to influence tumorigenic processes that include proliferation, differentiation, and apoptosis. Evidence is accumulating that suggests a role for FA in reducing colorectal carcinogenesis (2, 5). Results from several laboratories, including our own, have demonstrated that a diet deficient in FA may be associated with an increased risk of colon neoplasia (2, 5), whereas dietary supplementation of this nutrient may be chemopreventive (14, 15, 19).

However, the mechanisms by which dietary and/or supplemental FA modulates intestinal tumorigenesis are poorly understood. Recent in vitro studies from our laboratory have demonstrated that supplemental FA markedly inhibits proliferation of colon cancer cell lines and that this inhibition is accompanied by a concomitant reduction in expression and tyrosine kinase activity of EGFR. Because EGFR plays a critical role in regulating the growth of normal, preneoplastic, and neoplastic cells (8, 22), our observation of inhibition of expression and activation of EGFR in colon cancer cell lines by FA led us to postulate that FA may exert its growth-inhibitory property by attenuating the EGFR signaling processes. To test this hypothesis, we examined the effect of supplemental FA and its metabolites DF, TF, and 5-MTF on basal and serum-induced activation of the EGFR promoter in HCT-116 cells. Our present observation that exposure of HCT-116 cells to supplemental FA or any one of its metabolites inhibits EGFR transcription (as evidenced by inhibition of activity of the EGFR promoter luciferase construct pRE1102) suggests that FA and its metabolites exert a direct effect on the EGFR promoter. Moreover, the finding that FA and 5-MTF also inhibit the serum-induced activation of the EGFR promoter lends further support to the hypothesis that FA and 5-MTF modulate EGFR activity. This modulation of EGFR activity is evident not only for the 1,102-nt-long EGFR promoter pRE1102 but also for the construct where 260 nt were deleted (pRE1102Δ260) or for a construct that contains only 260 nt (pRE260) of the EGFR promoter sequence. The EGFR promoter fragments in pRE1102 and pRE260 contain multiple methylation sites, whereas pRE1102Δ260 contains one putative site for DNA methylation. These methylation sites are based on the consensus sequence (ccgg) for the restriction enzyme HpaII or MspI. Our observation that MTF inhibition of serum-induced activity of pRE1102 and pRE1102Δ260 is similar suggests that modulation of EGFR promoter activity by FA/MTF is independent of the number of promoter methylation sites. Furthermore, the fact that the extent of 5-MTF-dependent inhibition of serum-induced stimulation of pRE260 is similar to that of pRE1102Δ260, which lacks TCC and Sp1 motifs, suggests that FA/MTF-dependent regulation of EGFR is also independent of TCC and/or Sp1 motifs. Taken together, the results suggest that an excess of FA or its metabolites, specifically 5-MTF, can inhibit EGFR transcription, leading to decreased expression of the receptor in colon cancer cells. Moreover, the fact that exposure of HCT-116 cells to 5-MTF causes no inhibition of the serum-induced activation of c-fos promoter indicates that FA specifically represses EGFR transcription in colon cancer cells.

Although the exact mechanism(s) by which FA inhibits EGFR promoter activity is not fully understood, it is reasonable to speculate that hypermethylation of the EGFR could be partly responsible for this process. The basis for this postulation is that FA is the primary methyl donor for DNA methylation, which subsequently plays a critical role in modulating gene expression. DNA methylation is the addition of a hydrophobic methyl group to the cytosine in a CG sequence. Methylation of CpG sequences stabilizes chromatin, prevents binding of transcription factors, and is thought to be one of the primary mechanisms by which genes are turned on and off developmentally (3, 17, 21, 26, 28). Hypermethylation has also been shown to cause functional inactivation of a number of tumor suppressor genes (12). For example, in the breast cancer cell line MDA-MB-435, methylation of the promoter of connexin 26 has been shown to be associated with a concomitant loss of its expression (27). Hypermethylation has also been shown to cause functional inactivation of p16INK4A (25). Our observation of increased amplification of methylated EGFR promoter in MTF-treated HCT-116 cells suggests that FA induces methylation of the promoter sequences. This could
partly be responsible for FA-dependent inhibition of EGFR. Moreover, 5-aza-2’-deoxycytidine, an inhibitor of methylation, failed to abrogate serum-induced activation of the EGFR promoter luciferase reporter construct pRE1102-Luc. Taken together, these observations suggest that FA inhibition of EGFR involves increased EGFR promoter methylation.

In conclusion, our present data demonstrate that supplemental FA or any one of its metabolites, particularly 5-MTF, inhibits constitutive and serum-induced activity of the EGFR promoter, but not c-fos promoter activity, in HCT-116 colon cancer cells. Inhibition of methylation reverses the FA-induced inhibition of EGFR promoter activity. Our present data suggest that FA and its metabolite 5-MTF inhibit EGFR promoter activity in colon cancer cells by enhancing the expression of the promoter. This could partly be responsible for FA-mediated inhibition of growth-related processes in colorectal neoplasia.

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