Folic acid and its metabolites modulate IGF-I receptor gene expression in colon cancer cells in a p53-dependent manner

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Abstract

The insulin-like growth factor-I receptor (IGF-IR) has an important role in colorectal cancer development and progression. IGF-IR displays a potent anti-apoptotic activity and is overexpressed in primary tumors and colon cancer-derived cell lines. Folic acid, a member of the vitamin B family, is a chemopreventive agent whose deficiency has been linked to an enhanced colon cancer risk. The present study was aimed at testing the hypothesis that part of the modulatory effect of folic acid on malignant transformation may be attributed to its ability to regulate IGF-IR gene expression. Regulation of IGF-IR gene expression by folic acid was assessed using western blots, RT-PCR, transient transfections and chromatin immunoprecipitation assays. Activation of the IGF-IR signaling pathway was evaluated by measuring phosphorylation of ERK, and apoptosis was assayed using poly (ADP-ribose) polymerase cleavage and annexin V-FITC staining. Results obtained showed that folic acid induced a dose-dependent decrease in IGF-IR protein and mRNA levels in the HCT116 $+/+$ colon cancer cell line. This effect was associated with a significant reduction in IGF-IR promoter activity. Similar effects were elicited by the folic acid metabolites dihydrofolic acid and tetrahydrofolic acid. In addition, folic acid abrogated the IGF-I-stimulated phosphorylation of the downstream signaling molecule ERK1/2 and exhibited a pro-apoptotic activity. Moreover, folic acid induced a significant decrease in Sp1 binding to the IGF-IR promoter region. Finally, folic acid had no effect in wild-type p53-depleted HCT116 $-/-$ and Caco-2 cells. In conclusion, the mechanism of action of folic acid involves regulation of IGF-IR gene expression. The ability of folic acid to downregulate the IGF-I signal transduction pathway may allow the micronutrient to function as a chemopreventive agent. Folic acid deficiency, on the other hand, may lead to increased IGF-IR gene expression, with ensuing pathological activation by endocrine and/or autocrine/paracrine IGF-I.

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Introduction

Diseases of the colon and rectum have assumed the role of a major public health issue in the Western world. The potential involvement of specific micronutrients in colorectal cancer etiology has been the subject of extensive investigation. Folic acid, a member of the vitamin B family, has emerged in recent years as an important player in the etiology of digestive tract cancers, including tumors of the colorectum, esophagus and stomach (Duthie 1999, Kim 2003). Evidence of both an epidemiological and an experimental nature suggested a chemopreventive

role for folic acid in colorectal cancer (Giovannucci 2002). Thus, whereas a folic acid-deficient diet was shown to be associated with an increased risk of colon cancer (Giovannucci *et al.* 1998, Choi & Mason 2002), dietary folic acid supplementation provided significant protection against the disease (Lashner *et al.* 1997). The chemopreventive properties of folic acid may be attributed to: (i) its role in the process of DNA methylation, and (ii) its participation in the synthesis of purines and pyrimidines and, consequently, DNA synthesis and repair (Duthie 1999). Hence, folic acid deficiency may activate the tumorigenic process by altering

the expression of genes involved in critical cellular functions, including cell cycle control, cell death and DNA repair (Duthie 2001).

The insulin-like growth factor (IGF) system plays a critical role in growth and development of many tissues and is also thought to play a prominent role in tumorigenesis. IGF-I and IGF-II are potent mitogenic hormones that are mainly produced in the liver, although malignantly transformed colon cancer cells express significant levels of both ligands. Increased serum levels of IGF-I were recently found to be associated with increased occurrence of adenomatous polyps and even advanced adenomas. In addition, colon cancer cells express high levels of IGF-I receptor (IGF-IR), a tyrosine kinase-containing transmembrane receptor coupled to several intracellular second messenger pathways, including the *ras-raf*-MAPK and phosphatidylinositol 3-kinase-protein kinase B/Akt signaling cascades (LeRoith *et al.* 1995, Werner & LeRoith 1996, Sekharam *et al.* 2003). The central role of IGF-IR in colon cancer biology is illustrated by the results of experiments showing that IGF-IR blockade inhibited tumor growth and angiogenesis and, furthermore, enhanced chemotherapy-induced apoptosis (Adachi *et al.* 2002, Reinmuth *et al.* 2002). However, the transcriptional mechanisms and transcription factors that are directly responsible for IGF-IR overexpression in colorectal cancer cells have not yet been identified.

Owing to the fundamental role of IGF-IR action in colon cancer development, and in view of the chemopreventive function of folic acid, we hypothesized that folic acid may modulate proliferative processes via mechanism/s that involve regulation of IGF-IR gene expression. Results obtained show that folic acid downregulates IGF-IR promoter activity as well as endogenous IGF-IR mRNA and protein levels in a dose-dependent manner. Furthermore, the mechanism of action of folic acid involves inhibition of Sp1 binding to *cis*-elements in the proximal IGF-IR promoter region. In addition, folic acid inhibits the IGF-I-induced phosphorylation of downstream mediators, including the signaling molecule ERK, and exhibits a pro-apoptotic activity. Folic acid had no effect on IGF-IR gene expression in cells with a disrupted p53. Combined, our data suggest that the IGF-IR gene is a novel downstream target for folic acid action. The chemopreventive function of folic acid may be linked to its ability to downregulate the IGF-IR signaling pathway. Folic acid deficiency, on the other hand, may lead to aberrant IGF-IR gene expression and

enhanced receptor activation by locally produced and/or circulating IGF-I.

Materials and methods

Cell cultures

Human colorectal cancer cell lines HCT116 +/+, which expresses wild-type p53, and HCT116 -/-, in which the p53 gene has been disrupted by targeted homologous recombination, were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 50 µg/ml gentamicin sulfate. HCT116 cells were provided by Dr Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD, USA) (Bunz *et al.* 1998). The human colon cancer cell line Caco-2 was obtained from the American Type Culture Collection (Manassas, VA, USA). Caco-2 cells were maintained in RPMI-1640 medium containing 10% FBS.

Western blot analysis

Cells were serum-starved overnight, after which they were treated with increasing concentrations of folic acid. After 24 h, cells were harvested with ice-cold PBS containing 5 mM EDTA and lysed in a buffer composed of 150 mM NaCl, 20 mM Hepes, pH 7.5, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM polymethylsulfonyl fluoride, 2 µg/ml aprotinin, 1 mM leupeptin, 1 mM pyrophosphate, 1 mM vanadate and 1 mM dithiothreitol. Samples (80 µg protein) were subjected to 10% SDS-PAGE, followed by electrophoretic transfer of the proteins to nitrocellulose membranes. Membranes were blocked with 3% milk in T-TBS (20 mM Tris-HCl, pH 7.5, 135 mM NaCl, and 0.1% Tween-20) and then incubated with an anti-human IGF-IR β -subunit antibody (C20; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed extensively with T-TBS, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were detected using the SuperSignalWest Pico Chemiluminescent Substrate (Pierce). In addition, blots were probed with antibodies against total ERK, phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴) and tubulin.

RT-PCR

Total RNA was prepared from IGF-I-treated cultures using the AquaPure RNA isolation kit (Bio-Rad). IGF-IR mRNA levels were measured

by semiquantitative RT-PCR, using the following primers: sense, 5'-TGGAGTGCTGTATGCCTCT-G-3'; antisense, 5'-TGATGACCAGTGTGGCT-GG-3' (Lighten *et al.* 1997). The size of the amplified IGF-IR mRNA fragment was 329 bp. For control purposes, levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were measured using the following primers: sense, 5'-ACCACAGTCCATGCCATCAC-3'; antisense, 5'-TCCACCACCCTGTTGCTGTA-3' (Chen *et al.* 2004). The size of the amplified GAPDH mRNA fragment was 452 bp.

Quantitative real-time PCR

Quantitative real-time PCR was performed using TaqMan Universal PCR MasterMix and Assay-on-Demand Gene Expression primers and probes (Hs00609566_M1) (Applied Biosystems). An ABI Prism 7000 Sequence Detection System was employed. The GAPDH mRNA levels were analyzed as an internal control and used to normalize IGF-IR mRNA values. Amplification was carried out after an incubation of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The number of PCR cycles to reach the fluorescence threshold was the cycle threshold (C_t). Each cDNA sample was tested in triplicate and mean C_t values are reported. Furthermore, for each reaction, a 'no template' sample was included as a negative control. The relative expression of each mRNA was calculated by ΔC_t (where ΔC_t is the value obtained by subtracting the C_t value of GAPDH mRNA from the C_t value of the target mRNA). $\Delta\Delta C_t = (\Delta C_t \text{ of treated sample} - \Delta C_t \text{ of control})$. The relative quantification was determined by standard $2^{-(\Delta\Delta C_t)}$ calculations.

Plasmids and DNA transfections

Transient transfection experiments were performed using an IGF-IR promoter-luciferase reporter plasmid extending from nucleotide (nt) -476 to +640 (p(-476/+640)LUC; nt +1 corresponds to the transcription initiation site). The basal promoter activity of this fragment, containing most of the proximal IGF-IR promoter region, has been previously described (Werner *et al.* 1992, 1994). Transient transfections were also performed using deleted reporter constructs that include 188 or 40 bp of the IGF-IR 5'-flanking region (p(-188/+640)LUC and p(-40/+640)LUC respectively). HCT116 cells were transfected with 1 µg of the IGF-IR promoter-reporter

plasmid, along with 0.2 µg of a β -galactosidase plasmid (pCMV β), using the Metafectene reagent (Biontex Laboratories GmbH, Munich, Germany). Twenty-four hours after transfection, folic acid was added to the medium and cells were harvested after an additional 24 h. In some experiments, increasing doses of the folic acid metabolites dihydrofolic acid and tetrahydrofolic acid were added to the cultures. Luciferase and β -galactosidase activities were measured as previously described (Werner *et al.* 1992).

Chromatin immunoprecipitation (ChIP) analysis

Folic acid-treated cultures were incubated with formaldehyde (1% final concentration) for 10 min at room temperature. At the end of the incubation period, cells were washed twice and harvested using ice-cold PBS. Pelleted cells were resuspended in a 1% SDS-containing buffer, incubated on ice for 10 min, and sonicated for 3 min. Cell extracts were then immunoprecipitated with anti-Sp1 antibody for 18 h at 4°C. For PCR analysis of Sp1-immunoprecipitated chromatin, a set of primers encompassing the proximal human IGF-IR promoter (nt -486 to +287) was employed (Cooke *et al.* 1991). Sequencing and DNaseI footprinting analyses revealed the presence of four *cis*-elements for transcription factor Sp1 in this particular region (Beitner-Johnson *et al.* 1995). PCR was performed using the Thermal Ace DNA polymerase kit (Invitrogen).

Apoptosis measurements

HCT116 +/+ cells were serum-starved for 24 h, after which they were treated with increasing concentrations of folic acid (0, 10, 50 and 100 µg/ml), in the absence or presence of IGF-I (50 ng/ml). Cells (1×10^6) were washed in ice-cold PBS and resuspended in binding buffer (Annexin V-FITC kit; Bender Med Systems GmbH, Vienna, Austria). Annexin V-fluorescein isothiocyanate (FITC) was added to the cell suspension and incubated for 10 min in the dark. Propidium iodide (PI) was then added and stained cells were analyzed using a FACSsort Flow Cytometer (Beckton Dickinson). Viable cells are primarily Annexin V-FITC- and PI-negative; PI-positive staining indicates necrosis, Annexin V-FITC-positive staining indicates early apoptosis, and cells that are Annexin V-FITC- and PI-positive are considered to be in late apoptosis. Folic acid-induced apoptosis was also evaluated by western immunoblotting using a poly (ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology).

Results

IGF-IR overexpression is a typical hallmark of most types of cancer, including colorectal neoplasms. Because folic acid is a nutritional factor whose depletion has been associated with an increased risk for colorectal cancer development, we investigated the potential participation of this nutrient in the regulation of IGF-IR gene expression. Human colon

cancer-derived HCT116 +/+ cells were serum-starved overnight and then incubated with increasing concentrations of folic acid for 24 h. Western blot analysis using an anti-IGF-IR β -subunit antibody revealed that folic acid induced a dose-dependent decrease in endogenous IGF-IR levels. Maximal inhibition ($56.5 \pm 6.5\%$ of control) was seen at a folic acid dose of $100 \mu\text{g/ml}$ whereas no change was seen in the levels of tubulin (Fig. 1A and B). To

Figure 1 Regulation of IGF-IR gene expression by folic acid (F.A.). (A) Serum-starved HCT116 +/+ cells were incubated with increasing doses of folic acid for 24 h. At the end of the incubation period cells were collected as described in Materials and methods and equal amounts of protein (80 μg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. IGF-IR levels were determined using a polyclonal IGF-IR antibody, followed by incubation with an HRP-conjugated secondary antibody. The position of the 97 kDa IGF-IR β -subunit is indicated. Membranes were re-probed with a tubulin antibody. The figure shows the result of a typical experiment, repeated four times. (B) Bands corresponding to IGF-IR and tubulin were quantified using scanning densitometry and the optical density was expressed as IGF-IR values normalized to tubulin. A value of 100% was given to the IGF-IR/tubulin level in untreated cells. The bars represent means $\pm$ S.E.M. of four independent experiments. * $P < 0.01$ vs control cells. (C) HCT116 +/+ cells were treated with folic acid (10–50 $\mu\text{g/ml}$) for 24 h, or left untreated, after which total RNA was prepared and IGF-IR and GAPDH mRNA levels were measured by semiquantitative RT-PCR. 'M', molecular weight marker; 'RT-', control reaction in the absence of reverse transcriptase; 'C', control reaction in the absence of cDNA. (D) Scanning densitometry of IGF-IR mRNA levels normalized to GAPDH mRNA levels. Bars are means $\pm$ S.E.M. of three experiments. (E) Quantitative real-time PCR of IGF-IR mRNA levels in folic acid-treated HCT116 +/+ cells. Cells were treated with increasing doses of folic acid, after which total RNA was prepared and IGF-IR mRNA and GAPDH mRNA values were measured using the TaqMan Real-time PCR system. Analysis of the data was performed as described under Materials and methods. * $P < 0.01$ vs control cells.

Figure 2 Regulation of IGF-IR promoter activity by folic acid (F.A.). (A) Schematic representation of IGF-IR promoter–reporter constructs. Plasmids p(–476/+640)LUC, p(–188/+640)LUC and p(–40/+640)LUC contain, respectively, 476, 188 and 40 bp of 5′-flanking region (open bar) and 640 bp of 5′-untranslated region (closed bar) of the IGF-IR gene, fused to a luciferase cDNA (LUC). An arrow denotes the transcription ‘initiator’ element. The luciferase cDNA is not shown to scale. Triangles denote a cluster of four Sp1 sites. (B) HCT116 +/+ cells were transfected with 1 µg of the p(–476/+640)LUC IGF-IR promoter–luciferase reporter construct, along with 0.2 µg of the pCMVβ plasmid, using Metafectene reagent. Twenty-four hours after transfection, folic acid (0, 10, 50 or 100 µg/ml) was added to the cultures and cells were incubated for an additional 24 h, after which they were harvested and the levels of luciferase and β-galactosidase were measured. Promoter activities are expressed as luciferase values normalized for β-galactosidase levels. A value of 100% was given to the promoter activity in the absence of folic acid treatment. Results are means ± S.E.M. of four independent experiments, performed in duplicate dishes. **P* < 0.01 vs untreated cells. (C) Cells were transfected as indicated above and, after 24 h, increasing doses of dihydrofolic acid (open bars) or tetrahydrofolic acid (solid bars) were added to the culture medium. Cells were lysed after an additional 24 h. Results are means ± S.E.M. of three experiments, performed in duplicate plates. (D) HCT116 +/+ cells were transfected with 1 µg of the p(–476/+640)LUC reporter construct or IGF-IR-deleted plasmids (p(–188/+640)LUC or p(–40/+640)LUC) and 0.2 µg of the pCMVβ plasmid. Transfected cells were incubated with folic acid (50 µg/ml) (open bars), or left untreated (solid bars) and processed as described above. A value of 100% was given to the basal activity of each reporter construct in the absence of folic acid. **P* < 0.01 vs controls.

establish whether the inhibitory effect of folic acid was associated with a corresponding reduction in IGF-IR mRNA levels, HCT116 +/+ cells were treated with folic acid for 24 h, after which IGF-IR mRNA levels were measured by semiquantitative RT-PCR. Results obtained showed that folic acid induced a significant decrease in IGF-IR steady-state mRNA levels ($50.0 \pm 1.5\%$ of control at 10 µg/ml folic acid and $46.2 \pm 5.7\%$ at 50 µg/ml) (Fig. 1C and D). No change was seen in GAPDH mRNA levels. These results were corroborated by TaqMan real-time PCR. As shown in Fig. 1E, folic acid (50 µg/ml) induced a 40% reduction in IGF-IR mRNA values, while at 10 µg/ml there was a 10% inhibition. Further-

more, TaqMan analyses showed no reduction in IGF-IR mRNA levels at short incubation times (2–4 h) (data not shown).

To determine whether the effect of folic acid on IGF-IR gene expression was mediated at the level of transcription of the IGF-IR gene, transient transfection experiments were performed in HCT116 +/+ cells using a luciferase reporter construct under the control of the proximal IGF-IR promoter (p(–476/+640)LUC) (Fig. 2A). Twenty-four hours after transfection, increasing doses of folic acid were added to the cells and, after an additional 24 h, cells were collected and promoter activity was measured. Results obtained indicated that folic

acid repressed IGF-IR promoter activity, with maximal suppression achieved at a concentration of 50 µg/ml (66 ± 4.3% of control untreated cells) (Fig. 2B). Similar decreases in luciferase activity were elicited by the folic acid metabolites dihydrofolic acid and tetrahydrofolic acid (Fig. 2C). To more accurately map the IGF-IR promoter region responsible for mediating the effect of folic acid, transfections were performed using the deleted reporter plasmids p(−188/+640)LUC and p(−40/+640)LUC. Construct p(−188/+640)LUC lacks a cluster of four Sp1 sites between nt −399 and −331 that appears to mediate the majority of Sp1 activation of the promoter. Construct p(−40/+640)LUC includes a minimal 5′-flanking sequence composed of 40 bp located immediately upstream of the ‘initiator’ element (Beitner-Johnson *et al.* 1995). The ability of folic acid to inhibit IGF-IR promoter activity was abrogated in the p(−188/+640)LUC and p(−40/+640)LUC reporter plasmids in comparison with p(−476/+640)LUC (Fig. 2D).

Sp1 was previously identified as a zinc-finger nuclear protein which plays a crucial role in IGF-IR gene transactivation. To examine whether folic acid treatment may lead to reduced Sp1 binding to *cis*-elements in the IGF-IR promoter region, ChIP experiments were performed using folic acid-treated HCT116 +/+ cells (or control untreated cells). At the end of the 24-h incubation period, cell lysates were immunoprecipitated with anti-Sp1, after which precipitated chromatin was amplified by PCR using primers encompassing the proximal human IGF-IR promoter. Results of ChIP assays showed that folic acid (50 µg/ml) induced a noticeable decrease in Sp1 binding to the IGF-IR promoter (Fig. 3).

To examine whether folic acid can modulate early IGF-I-mediated signal transduction events, HCT116 +/+ cells were incubated for 24 h in the absence or presence of folic acid (10 µg/ml) and then treated with IGF-I (50 ng/ml) for 20 or 40 min. Phosphorylation of the downstream signaling molecule ERK was assessed using a specific anti-phospho-ERK1/2. As shown in Fig. 4, folic acid treatment abrogated the IGF-I-stimulated ERK1/2 phosphorylation at both 20 and 40 min, although results reached statistical significance only at 20 min of IGF-I treatment ($P < 0.01$ vs IGF-I-treated cells in the absence of folic acid).

Previous studies have established that folic acid inhibits colon cancer cell proliferation and enhances epithelial apoptosis (Jaszewski *et al.* 1999, Akoglu *et al.* 2001, 2004, Duthie 2001, Cao *et al.* 2005). To

Figure 3 Chromatin immunoprecipitation assay. HCT116 +/+ cells were incubated with increasing doses of folic acid (F.A) for 24 h. After incubation, cells were lysed and immunoprecipitated with an anti-Sp1 antibody (α sp1), followed by PCR amplification of precipitated chromatin using primers encompassing the human IGF-IR promoter region extending from −486 to +287. The position of the 773 bp amplified fragment is denoted by the arrow. The input band represents the amplified PCR product in the absence of Sp1 antibody.

Figure 4 Effect of folic acid (F.A) on IGF-I-stimulated signal transduction. (A) HCT116 +/+ cells were incubated with folic acid (10 µg/ml) for 24 h (or left untreated) and then treated with IGF-I (50 ng/ml) for 20 or 40 min. Cell lysates were electrophoresed, transferred to membranes, and probed with antibodies against phospho (p)-ERK1/2 and total ERK1/2. (B) The bar graph denotes the densitometric scanning of the phospho-ERK1/2 bands normalized to the corresponding total ERK1/2 band. The bars represent the means ± S.E.M. of three independent experiments. * $P < 0.05$ vs unstimulated cells (lanes 2 vs 1); ** $P < 0.05$ vs IGF-I-treated cells (lanes 4 vs 2).

Figure 5 Analyses of the effect of folic acid (F.A) on apoptosis. (A) Serum-starved HCT116 $+/+$ cells were treated with folic acid (50–100 $\mu\text{g/ml}$) for 24 h (or left untreated, lane 1), in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of IGF-I (50 ng/ml) during the last 1 h of the incubation period. Apoptosis was evaluated by western blotting using anti-PARP. Membranes were re-probed with a tubulin antibody. (B) Apoptosis was assessed in folic acid-treated and control HCT116 $+/+$ cultures using an Annexin-FITC kit, as described in Materials and methods. The horizontal axis denotes ‘Annexin V-FITC positive cells’ and the vertical axis represents ‘PI positive cells’. Results of a representative experiment repeated three times are shown. (C) Quantitative analysis of the flow cytometry data was performed using WinMDI 2.8 software (<http://facs.scripps.edu/software.html>).

examine whether folic acid exhibits a pro-apoptotic activity under our experimental conditions, HCT116 $+/+$ cells were serum-starved overnight, after which they were incubated with folic acid (50–100 $\mu\text{g/ml}$) for 24 h and apoptosis was evaluated by Western blotting using an anti-PARP antibody. As shown in Fig. 5A, lanes 2 and 3, folic acid induced the appearance of an ~ 85 kDa protein, which represents a cleavage product of the ~ 116 kDa PARP protein, and which is considered

a marker of early apoptosis. These results were corroborated using an Annexin V-FITC kit. Results of FACS analysis showed that folic acid enhanced apoptosis in a dose-dependent fashion ($20.5 \pm 3.1\%$ apoptotic cells at 50 $\mu\text{g/ml}$ folic acid and $22.3 \pm 4.2\%$ apoptotic cells at 100 $\mu\text{g/ml}$, compared with $13.2 \pm 2.4\%$ apoptotic cells in control cultures) (Fig. 5B and C). To determine whether IGF-I can abolish the pro-apoptotic action of folic acid, cells were incubated with folic acid in the

presence of IGF-I (50 ng/ml, 1 h), after which apoptosis was measured. Western blotting of PARP protein revealed that the intensity of the 85 kDa band was significantly diminished in cells that were treated with both IGF-I and folic acid, compared with cells treated only with folic acid (Fig. 5A, compare lanes 4 vs 2 and 5 vs 3).

Finally, to examine the potential contribution of p53 status to folic acid action, IGF-IR levels were measured in folic acid-treated HCT116 $-/-$ cells, in which the p53 gene has been disrupted by homologous recombination. The rationale for this experiment was the fact that folic acid has been recently shown to enhance p53 expression in gastric mucosa (Cao *et al.* 2005), suggesting that p53 may mediate the inhibitory action of folic acid. Results of western immunoblots showed that folic

acid had no effect on IGF-IR levels in p53-depleted HCT116 $-/-$ cells (Fig. 6A). Moreover, folic acid had no major effect on IGF-IR promoter activity in HCT116 $-/-$ cells (Fig. 6B). Likewise, we were unable to see a folic acid-induced decrease in IGF-IR levels in the human colon cancer cell line Caco-2, which includes a mutant p53 allele (Fig. 6C). Finally, we were unable to detect an increase in p53 levels in folic acid-treated HCT116 $+/+$ cells (data not shown).

Discussion

The important role of the IGF system in colon cancer has been firmly established. Circulating IGF-I levels were shown to control the incidence of tumor development and hepatic metastasis in a mouse model of colon cancer as well as the incidence of adenomatous polyps in humans (Wu *et al.* 2002). IGF-IR, which mediates the anti-apoptotic activity of the IGF ligands, is abundantly expressed in colon cancer cells. Furthermore, specific blockade of IGF-IR was shown to be associated with inhibition of tumor growth and angiogenesis, and with enhanced chemotherapy-induced apoptosis (Adachi *et al.* 2002, Reinmuth *et al.* 2002). While nutritional status has a profound impact on IGF-IR gene expression and action, the contribution of specific micronutrients has not yet been explored in a systematic manner. Folic acid, a water-soluble member of the vitamin B family found in fruits and green leafy vegetables, exhibits a chemoprotective action that has been linked to its capacity to affect DNA methylation and/or normal DNA synthesis and repair. Poor folate intake may lead to inappropriate proto-oncogene activation, aberrant DNA repair, and chromosomal damage (Duthie 1999). We chose therefore to test the hypothesis that folic acid may act through modulation of IGF-IR gene expression.

The results presented here demonstrate that physiological concentrations of folic acid induced a dose-dependent decrease in endogenous IGF-IR protein and mRNA levels (Fig. 1), suggesting that the IGF-IR gene is a relevant target for folic acid action. Furthermore, the inhibitory action of folic acid and two of its metabolites is mediated at the transcriptional level, as demonstrated by the results of transient transfection experiments (Fig. 2). Molecular characterization of the IGF-IR gene regulatory region revealed that the IGF-IR promoter includes a unique initiator motif from which transcription starts *in vivo* and, similarly to other growth factor

Figure 6 Effect of p53 status on folic acid (F.A) action. (A) p53-depleted HCT116 $-/-$ cells were serum-starved overnight, after which they were treated with increasing concentrations of folic acid for 24 h. IGF-IR abundance was assessed by western immunoblotting as indicated in Materials and methods. (B) HCT116 $-/-$ cells were transfected with 1 µg of the p(-476/+640)LUC IGF-IR promoter-luciferase reporter construct, along with 0.2 µg of pCMVβ. After 24 h, folic acid (0, 10, 50 or 100 µg/ml) was added to the cultures and cells were incubated for an additional 24 h. Cells were harvested and luciferase and β-galactosidase activities were determined. Results are means ± S.E.M. of three independent experiments. (C) Serum-starved Caco-2 cells were treated with folic acid for 24 h and IGF-IR levels were measured by western blotting as described above.

receptor-encoding genes, contains multiple binding sites (GC boxes) for members of the Sp1 family of zinc-finger transcription factors (Courey & Tjian 1988, Werner *et al.* 1992, Beitner-Johnson *et al.* 1995). Results of ChIP experiments presented here suggest that a potentially novel mechanism of action of folic acid may involve a decrease in Sp1 binding to *cis*-elements in the proximal IGF-IR promoter region. Reduction in Sp1 binding has been previously shown to lead to diminished IGF-IR gene transcription (Abramovitch *et al.* 2003). The finding that folic acid abrogated the IGF-I-stimulated increase in ERK phosphorylation may be consistent with the possibility that folic acid controls IGF-IR levels and IGF-IR action at both transcriptional and post-transcriptional levels. Recent studies showed that folic acid increased IGF-binding protein-3 (IGFBP-3) levels in several cervical cancer cell lines. These results suggest that folic acid may modulate IGF action by concomitant inhibition of the anti-apoptotic IGF-IR gene and stimulation of the pro-apoptotic IGFBP-3 gene (Mathur & Mathur 2003).

A recent study has shown that folic acid and its metabolites inhibit basal and serum-stimulated epidermal growth factor receptor (EGFR) promoter activity (Nagothu *et al.* 2004). In contrast to our results, the repression of the EGFR promoter by folic acid seems to be Sp1-independent as Sp1-deleted EGFR reporter constructs were inhibited by folic acid to a similar extent as the wild-type reporter. Furthermore, the fact that 5-aza-2'-deoxycytidine (a methylation inhibitor) reversed the effect of folic acid on EGFR promoter activity, suggested that folic acid may repress EGFR gene expression by enhancing promoter methylation. While the IGF-IR gene promoter contains a number of CpG islands, no study so far has demonstrated methylation of the IGF-IR gene as a potential epigenetic mechanism for gene silencing. Combined, these studies suggest that folic acid exerts a chemopreventive role by governing expression of various growth factor receptor genes via multiple mechanisms of action.

In addition, the results of the present study demonstrate that folic acid repressed IGF-IR gene expression in a p53-dependent manner. Tumor suppressor p53 is a pivotal player in the apoptotic process and it has a major role in the protection of cells from DNA damage. The finding that folic acid was unable to downregulate IGF-IR levels in wild-type p53-depleted cells suggests that an intact p53 signaling pathway is a prerequisite to elicit the

pro-apoptotic effect of folic acid. Furthermore, since previous studies have demonstrated that wild-type, but not mutant, p53 suppressed IGF-IR promoter activity, we may speculate that the mechanism of action of folic acid involves stimulation of expression and/or activation of p53, which can then directly act at the IGF-IR promoter level (Werner *et al.* 1996). Consistent with this hypothesis, previous studies have demonstrated that folic acid enhances p53 expression in the gastric mucosa (Cao *et al.* 2005). Unfortunately, we were unable to demonstrate an increase in p53 levels in HCT116 +/+ cells following folic acid treatment. Interestingly, recent studies have identified the IGF-IR gene as a downstream target in a DNA-damage response pathway, whereas Sp1 was shown to mediate the IGF-IR response following ionizing radiation (Shahrabani-Gargir *et al.* 2004). In view of the important body of evidence indicating that folate deficiency is associated with DNA strand breaks, impaired DNA repair and increased susceptibility to mutagenesis, it is reasonable to suggest that at least part of the pathological spectrum associated with folate depletion may result from aberrant activation of the IGF-IR gene.

In conclusion, we have presented evidence showing that folic acid governs IGF-IR gene expression in colon cancer cells via mechanism/s involving transcriptional suppression of the IGF-IR promoter. These results are consistent with the notion that the chemopreventive function of folic acid may be linked to its ability to inhibit IGF-IR levels. Below a certain IGF-IR threshold level, the capacity of most cells to engage in mitogenic activity is significantly impaired (Rubini *et al.* 1997). Deregulated expression of the IGF-IR gene as a result of deficient folate intake may be associated with genomic instability, defective cell division, impaired checkpoint arrest, and enhanced transforming capacity.

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