Intestinal Neoplasia Induced by Low Dietary Folate Is Associated with Altered Tumor Expression Profiles and Decreased Apoptosis in Mouse Normal Intestine1–3

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Abstract

Epidemiological studies suggest that low dietary folate increases risk for intestinal neoplasia. We recently developed a unique tumor model in which mice fed low dietary folate developed DNA damage and intestinal tumors. To identify additional mechanisms by which reduced folate intake contributes to tumor formation in this model, we characterized gene expression signatures in tumors. A total of 175 probe sets had altered expression, with the majority (173) showing increased expression compared with normal intestine. Functional categorization revealed that most genes were involved in cancer (51 genes) or cell death (37 genes); 31 genes appeared in both categories. Because apoptosis resistance is a hallmark of neoplasia, we assessed apoptosis in normal intestine of mice fed control (CD) and low-folate diets (FD); apoptosis was reduced in FD normal intestine compared with CD intestine by active caspase-3 expression (P = 0.027) and caspase-3/7 activities (P = 0.059). We selected candidate genes with antiapoptotic properties that had increased expression in tumor microarrays, CD44, and gelsolin and confirmed these increases at the mRNA and protein levels. CD44 and gelsolin protein also increased in mice fed the FD compared with the CD, normal intestine. Bcl-2-like 1:Bcl-2-antagonist/killer 1 mRNA ratios tended to be greater in FD than in CD normal intestine (P = 0.056). In conclusion, tumors induced by low dietary folate exhibited gene expression profiles that are characteristic of disrupted apoptosis. Folate depletion in normal intestine may trigger neoplasia through increased DNA damage and defective apoptosis; upregulation of CD44 and gelsolin, and the mitochondrial apoptotic pathway are implicated. J. Nutr. 139: 488–494, 2009.

Introduction

Colorectal cancer is one of the most common malignancies in Western countries and represents the second leading cause of cancer death in the United States. Several factors can affect the transition from normal colonic mucosa to adenoma and carcinoma. Environmental factors characteristic of a Western lifestyle have been implicated in malignant transformation. In this regard, low dietary folate is strongly correlated with increased colon cancer risk (1). The folate metabolic pathway and, indirectly, cancer risk are influenced by diet, alcohol consumption, nutrient malabsorption, and genetic variants in key folate-metabolizing enzymes such as the common 677C→T mutation of methylenetetrahydrofolate reductase (MTHFR).4 Although the mechanisms by which alterations in folate metabolism contribute to cancer risk have not been clearly elucidated, it has been suggested that folate deficiency induces cancer by aberrant DNA methylation and by DNA damage through uracil misincorporation and chromosome breaks (2,3). Folate is required to generate 5-methyltetrahydrofolate, which provides one-carbon units for synthesis of S-adenosylmethionine, the universal methyl donor in many methylation reactions, including DNA and protein methylation. Folate is also required for the conversion of dUMP to dTMP for DNA synthesis and repair. MTHFR maintains the balance between folate utilized in DNA synthesis and methylation, because it catalyzes the conversion of 5,10-methylenetetrahydrofolate, the methyl donor in dTMP synthesis, to 5-methyltetrahydrofolate. In a previous study, we demonstrated that low dietary folate alone can induce intestinal tumors in mice without
Colon carcinogenesis involves molecular changes in cell differentiation, proliferation, and apoptosis. The intestinal epithelium undergoes rapid and continuous cell renewal from the stem cells in the lower regions of the crypts to the villus, a process controlled by apoptosis. Altered rates of epithelial cell apoptosis are thought to play an important role in colorectal carcinogenesis and progressive inhibition of apoptosis has been described during the transformation of colorectal epithelium to carcinoma (7–9).

Some studies have reported an enhancement of apoptosis after folate depletion in the media of cultured cells (2,10), but there is little information linking low folate with apoptosis in vivo. Liu et al. (11) reported a decrease in apoptosis in mouse colon after dietary depletion of several vitamins, including folate; however, no significant changes were found due to low folate alone.

An alteration of the balance between DNA damage and cell death might lead to enhanced tumorigenesis in colon cancer. Because we have already demonstrated that our mouse model shows increased DNA damage, we investigated whether decreased apoptosis was also involved in tumor formation in these mice. Toward this end, we characterized the gene expression signatures of folate-dependent intestinal tumors and the effect of low dietary folate on apoptosis in the normal intestine of our mice. Functional analysis of differentially regulated genes between tumors and normal intestine showed a striking number of candidate genes involved in the regulation of apoptosis. These included: CD44, a stem cell marker, whose expression has been linked to a decrease in apoptosis of colon cancer cells (12) and of murine normal intestinal epithelium (13), and gelsolin (Gsn), an actin regulatory protein with apoptosis inhibitory properties (14,15). Both of these genes were upregulated and may be involved in decreased apoptosis and tumor formation in our dietary model.

**Materials and Methods**

**Mice and diets.** Animal experimentation was approved by the Montreal Children’s Hospital Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. After weaning, Mthfr<sup>+/−</sup> and Mthfr<sup>−/−</sup> BALB/c mice, previously generated in our laboratory (16), were fed a control diet (CD) or a folate-deficient diet (FD) as previously described (6). All diets were supplemented with 1% succinylsulfathiazole to inhibit folate synthesis by intestinal flora. After 1 y, mice were killed and examined for intestinal tumors, as described (4).

**Microarray analysis.** Tumors and adjacent normal duodenum were isolated from Mthfr<sup>−/−</sup> mice fed the FD. Laser capture microdissection was performed to capture malignant and adjacent normal intestine. RNA extraction from captured cells was subsequently performed as previously described (4). RNA from individual samples was hybridized to Affymetrix Mouse 430.2 microarray chips at the McGill University-Genome Quebec Innovation Centre (Montreal, Canada). Normalized data were imported into GeneSpring Gx version 7.3.1 (Agilent Technologies), where each sample (normal and tumor) was normalized again to the corresponding normal intestine sample from that mouse (17). The fold of the normal intestine value was calculated for each probe set as the intensity of each tumor sample divided by the intensity of the matched normal intestine sample, averaged over all 5 pairs. Filter on confidence and filter on fold change functions were used to identify genes with significant differences in expression. A list of 175 probes met these criteria. Further analysis was performed with Ingenuity Pathways Analysis tool version 5.5.1–1002 (Ingenuity Systems) to categorize the genes into their appropriate biological function. We also compared our results with microarrays of mouse models of intestinal adenoma and with human studies in which normal tissues were compared with adenomas (18–21).

**Quantitative real-time RT-PCR.** RNA extraction and cDNA synthesis from normal intestine and tumors were performed as previously described (4). Primers for myelocytomatosis oncogene (Myc), tumor necrosis factor receptor superfamily member 12a (Tnfrsf12a), CD44 antigen (CD44 common exon), gelsolin (Gsn), tumor necrosis factor receptor superfamily member 6 (Fas), Fas ligand (Fasl), B-cell CLL/lymphoma 2 (Bcl-2), Bcl-2-like 1 (BclXL), Bcl-2-associated X protein (Bax), Bcl-2-antagonist/killer 1 (Bak), and gyceraldehyde-3-phosphate dehydrogenase genes were designed using Primer3 software (Supplemental Table 1). After RT, 20 ng of cDNA from the same cDNA batch was subjected to real-time PCR to amplify all genes in triplicate in a total reaction volume of 20 μL using Platinum SYBR Green Master mix, ROX as internal reference dye (Invitrogen), and the required amount of forward and reverse primers (Supplemental Table 1). Reactions were conducted on an Mx3000P thermocycler (Strategene) using the following cycling conditions: 95°C for 10 min and 40 cycles at 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s. For each experiment, a nontemplate reaction served as negative control. Melting curve analysis of the products as well as ampicol size verification on a 3% agarose gel confirmed the specificity of the PCR. The raw expression level for each gene was calculated using the same external standard curve made with a mixture of cDNA samples. Target gene expression was normalized to gyceraldehyde-3-phosphate dehydrogenase.

**Immunofluorescence.** Normal intestine and tumor immunofluorescence was performed as previously described (4). Sections were incubated overnight at 4°C with primary antibody: 1:100 rat anti-mouse CD44 antigen (BD Pharmigen), or 1:100 goat anti-human Gsn (Santa Cruz Biotechnology). Qualitative comparison between the immunostaining intensity of each tumor and normal adjacent tissue was performed.

**Apoptosis assay.** Caspase-Glo 3/7 activity (Promega) was used as an indicator of apoptosis. Normal intestine was ground into powder in liquid nitrogen and lysed in buffer (50 mmol/L KPO<sub>4</sub>, 0.3 mmol/L EDTA, pH 8.0). Total protein concentration was determined using BioRad. Two micrograms total protein was used, according to the manufacturer’s instructions.

**Western blotting.** Fifty micrograms protein from the same extracts used for Caspase-Glo 3/7 activity was separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes at 70 V, 2 h, 4°C. Nonspecific binding sites were blocked overnight at 4°C with 5% skim milk powder in TBS-Tween buffer. Primary antibodies and dilutions were: 1:100 rabbit anti-human active caspase-3 (Chemicon International), 1:10,000 rabbit anti-human β-actin (Sigma), 1:1000 rabbit anti-human CD44α (Santa Cruz Biotechnology), 1:1000 goat anti-human Gsn (Santa Cruz Biotechnology), 1:1000 rabbit anti-human BclXL (BD Pharmingen), and 1:2000 rabbit anti-human Bak (Upstate Biotechnology). Secondary antibodies were peroxidase-coupled anti-rabbit IgG (Amersham Biosciences) or donkey anti-goat IgG (Santa Cruz Biotechnology), as appropriate. Bands were detected with ECL Plus chemiluminescence system (Amersham Biosciences) and exposure to films. Band intensity was quantified with Quantity One v 4.1.0 software (BioRad). To ensure uniformity in comparisons of samples from different immunoblots, target protein/β-actin ratios were normalized relative to the mean ratio of the same 3 reference samples analyzed in each immunoblot.

**Statistical analysis.** We used 2-factor ANOVA to evaluate the effects of diet and Mthfr genotype and their interaction. Levene’s test was used for equality of variances. Pearson correlation was used for bivariate correlation analyses. All analyses were performed using SPSS for
Results

Microarray analysis of tumors compared with normal intestine in FD mice. The analysis revealed 175 probe sets in which the expression was altered in tumor tissue compared with normal intestine; this list should be considered preliminary until additional analyses are performed for confirmation. In the vast majority of these probes (173/175), increased expression was observed (Supplemental Table 2). These probe sets included 122 known genes, 4 expressed sequence tags, 24 RIKEN cDNA, and 6 unknown transcripts. Validation of the microarray expression differences by quantitative real-time RT-PCR (QRT-PCR) was conducted in 5 tumor samples from 5 mice on Myc and Tnfrsf12a. An increase to 11.34-fold of normal intestine for Myc expression ($P = 0.011$) and to 6.26-fold of normal intestine for Tnfrsf12a ($P = 0.023$) confirmed the microarray results.

We then categorized the genes in this list according to their biological functions (Fig. 1). The top 2 categories that best characterized our probe sets were cancer and cell death. Thirty-one genes were found in both cancer and cell death categories (Supplemental Table 3). An additional 20 and 6 genes, respectively, were present exclusively in the cancer category or the cell death category.

We also compared the results of our analysis with other previously published microarray studies of colorectal or intestinal tumors to determine whether some of the expression differences we observed had been reported by other groups (18–21) (Supplemental Table 3). There were no genes in our study and in both human adenoma studies in these 2 categories: Gsn and Interleukin 1 receptor like 1. The Interleukin 1 receptor like 1 gene was the only gene increased in expression in all 5 microarray data sets.

For further analysis, we selected 2 candidate genes associated with decreased apoptosis that could contribute to tumorigenesis in our mouse model: CD44 and Gsn. Gsn was significantly increased in adenomas in Alon et al. (1999) and Notterman et al. (2001) (18,19). Gene expression validation was conducted on 5 tumors compared with normal intestine by QRT-PCR. CD44 and Gsn expression in tumors showed 15.8 ($P = 0.019$) and 7.3 ($P = 0.002$) fold increases of normal intestine, respectively. We also examined protein levels by immunofluorescence and found an increase in CD44 and Gsn staining in 4 tumors compared with matched normal intestine (Fig. 2).

Apoptosis in normal intestine. To determine whether apoptosis in normal intestine is affected by low dietary folate, we measured active caspase-3 expression and the activities of the executor caspases 3/7 as biological indicators of apoptosis. A total of 24–30 mice (6–9 mice per group; Mthfr+/- and Mthfr+/+ mice fed CD and FD) were analyzed. There was a decrease in active caspase-3 expression due to folate deficiency ($P = 0.030$) (Fig. 3). Caspase-3/7 activity tended to be lower ($P = 0.059$) in normal intestine of the FD group [24,400 ± 3488 relative units (RU)] than in the CD group (38,033 ± 5902 RU). The difference was not significant, in contrast to the findings for active caspase-3, possibly because of the background noise induced by the presence of caspase-7 in the assay. However, in an analysis of 15 mice in which both assays were performed, we found a strong positive correlation between the 2 techniques ($P = 0.001; r = 0.761$). FD mice that formed tumors ($n = 6$) tended ($P = 0.145$) to have lower levels of apoptosis (0.41 ± 0.07) in normal intestine compared with the CD group ($n = 11; 0.64 ± 0.11$) in the active caspase-3 expression assay. This also was the case in the caspase-3/7 activity assay (25,391 ± 5591 vs. 38,033 ± 5902 RU; $P = 0.193$). Sample numbers were too small to detect significant differences. Apoptosis did not differ due to Mthfr genotype. These results suggest that low dietary folate is associated with decreased apoptosis in normal intestine.

Localization of apoptotic cells in the intestinal epithelium was performed using active caspase-3 immunostaining. Apoptotic cells were located at the top of the crypts, whereas there was no staining at the base of the crypts (data not shown).

Effect of FD on expression of apoptosis-related candidate genes CD44 and Gsn in normal intestine. We used Western blotting to measure CD44 antigen and Gsn protein levels in the same extracts from a total of 24–30 mice (6–9 mice per group; Mthfr+/- and Mthfr+/+ mice fed CD and FD). The FD group had greater levels of CD44 antigen ($P = 0.029$) (Fig. 4A) and Gsn protein ($P = 0.038$) (Fig. 4B) than the CD group. The analysis of CD44 and Gsn mRNA levels did not reveal differences due to folate deficiency. However, Gsn mRNA levels were higher in the 2 mice in the FD group with tumors (7.06 and 5.02) than in the CD group (4.51 ± 0.49; $n = 12$). There were no differences due to Mthfr genotype.

Analysis of apoptotic factors. To determine which apoptosis-related genes are involved in the resistance to apoptosis in FD
normal intestine, we performed QRT-PCR to measure the expression of several genes involved in apoptosis and colon cancer: *Fas*, *FasL*, *p53*, *Bcl-2*, *BclXL*, *Bax*, and *Bak*.

The analysis of 24 mice (6 mice per group; *Mthfr*1/1 and *Mthfr*1/2 mice fed CD and FD) revealed no differences at the mRNA level of any individual gene. However, *BclXL*:Bak mRNA ratios tended to be greater ($P = 0.056$) in the FD (1.66 ± 0.10) than in the CD group (1.40 ± 0.09). In the immunoblot analysis of the gene products, BclXL:Bak protein ratios tended to be greater ($P = 0.077$) in the FD group (1.67 ± 0.42) than in the CD group (0.80 ± 0.14) only in *Mthfr*1/1 mice by independent sample t test. In a qualitative analysis of BclXL:Bak protein ratios in individual mice, 9 of 12 mice in the FD group had higher protein ratios than the CD group. Although normal intestine was available from only 2 mice that had formed tumors, we observed higher BclXL:Bak mRNA ratios (2.13 and 1.68) compared with mice in the CD group (1.40 ± 0.09). There were no differences due to *Mthfr* genotype.

**Discussion**

In the initial report describing our tumor model, we suggested that the increase in DNA damage due to folate deficiency was an important contributor to tumorigenesis (4). Comparison of strain differences in tumorigenicity supported the influence of DNA damage on tumor formation (6). In this study, we continue to characterize mechanisms of tumorigenesis and identify a disruption in apoptosis as another major contributor to tumor formation following folate deficiency. Using gene expression profiling and functional characterization of gene expression differences in tumors, we observed that the majority of genes with changes were involved in cancer, cell death, or both. Four genes in the cancer or cell death categories were confirmed to be upregulated in tumors: *Myc*, *Tnfrsf12a*, *CD44*, and *Gsn* (22–25). It is therefore possible that alterations in apoptosis play a key role in the initiation and growth of tumors in our model.

Apoptosis is particularly critical in colorectal cancer due to the high proliferation rate of intestine (7–9,26). The analysis of normal mucosal biopsies from patients with resected adenomas revealed decreased apoptosis compared with mucosa of patients without a history of adenomas, suggesting a predisposing environment for malignant transformation (7,26). Studies on the contribution of folate deficiency to colon carcinogenesis have focused primarily on DNA damage and methylation differences. A few studies have examined the impact of folate deficiency on apoptosis in vitro (2). This phenomenon might be beneficial in eliminating potential tumorigenic cells. However, it may also increase the cell turnover rate and decrease repair time, thereby increasing risk for malignant transformation (2). In cancer cell lines or in cells with malignant transformation potential, folate deficiency may increase apoptosis (27–29).

Based on our findings, we suggest that low dietary folate negatively regulates apoptosis in normal intestine in vivo. Another study demonstrated decreased apoptosis in mouse intestine following multi-

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**FIGURE 3** Active caspase-3 protein levels in normal intestine of mice fed CD or FD. Values are means ± SEM, n = 6–9. Means without a common letter differ, $P < 0.05$. 

**FIGURE 2** CD44 (A) and Gsn (B) immunofluorescence in tumors and normal intestine. A representative experiment from 4 mice is shown at 40× magnification. Anti-CD44 (A, column 2) and anti-Gsn (B, column 2) were used to immunostain paraffin-embedded sections of tumors (top rows) and FD normal intestine from the same mouse (bottom rows). Column 1, Propidium iodide was used as a nuclear counterstain. AxioVision 40 software was used to merge the propidium iodide signal with the immunofluorescence signal (column 3). Column 4, Negative controls containing secondary antibody, but not primary antibody, for each sample. For both proteins, the tumors showed increased staining compared to normal intestine. FDT, FD diet tumor; FDN, FD diet normal intestine.
ple vitamin depletion. Although there were no differences due to folate deficiency alone, the lack of effect of folate alone could have been due to the relatively short deficiency period (10 wk) compared with our long-term study (11). Alternatively, the differences in the 2 studies may relate to the background strain of the mice, because we have shown that C57Bl/6 mice, the strain in the Liu et al. study (11), are resistant to tumorigenesis due to low folate (6).

Based on our interesting observation of decreased apoptosis, in the context of the increased DNA damage due to folate deficiency, we suggest that insufficient apoptosis in those damaged cells might increase the potential for uncontrolled cell proliferation and, eventually, transformation to adenoma and carcinoma. The localization of active caspase-3 at the surface of the crypts suggests that the decrease in apoptosis might occur in the differentiated cells of the villus. This hypothesis is consistent with the “top-down” model of colorectal carcinogenesis where the differentiated cells of the villus. This hypothesis is consistent with the “top-down” model of colorectal carcinogenesis where the differentiated cells of the villus. This hypothesis is consistent with the “top-down” model of colorectal carcinogenesis where the differentiated cells of the villus. This hypothesis is consistent with the “top-down” model of colorectal carcinogenesis where the differentiated cells of the villus. This hypothesis is consistent with the “top-down” model of colorectal carcinogenesis where the differentiated cells of the villus. This hypothesis is consistent with the “top-down” model of colorectal carcinogenesis where the differentiated cells of the villus. This hypothesis is consistent with the “top-down” model of colorectal carcinogenesis where

The mechanism by which low folate promotes resistance to apoptosis is unclear, although methylation differences could alter expression of critical apoptotic regulatory molecules. Apoptosis is a dynamic interaction of a complex network of proapoptotic (Bak, Bax, or Bad) and antiapoptotic (Bcl-2, BclXL, or Mcl1) factors affecting the relative permeability of the mitochondrial membrane to release cytochrome c and, ultimately, active caspase-3. We analyzed the effect of folate deficiency on mRNA levels of Fas receptor, Fasl, p53, Bcl-2, BclXL, Bax, and Bak genes in the normal intestine of our mouse model. Expression of individual genes was not altered. Instead, we found an increase in the BclXL:Bak mRNA and protein ratios in the normal intestine due to the FD diet. The protein analyses did not reach significance (P = 0.077); this may relate to the limitations of the immunoblotting method, which is more variable and possibly less sensitive than mRNA quantitation. However, a small increase in the BclXL:Bak ratio can be translated into a significant decrease of caspase-3 activity, because the mitochondrial pathway amplifies the apoptotic signal. Consequently, the decrease in apoptosis in normal intestine after folate depletion might be due to an increase in the BclXL:Bak ratio. Previous studies have reported an association between an increase in the BclXL:Bak ratio and human colorectal tumorigenesis or cancer progression (32,33). Colon cancer cells can also exhibit resistance to apoptosis through mutations in p53 or Apc (31,34), a decrease in Fas receptors (35), or tumor necrosis factor ligand superfamily member 10 (TRAIL)-mediated resistance (36). Although we did not find any differences in p53 or Fas receptor mRNA, alteration at the protein level of these genes or in other apoptotic regulatory molecules cannot be excluded. We chose to assess apoptosis in normal (preneoplastic) intestine to determine whether low folate creates a favorable environment for tumorigenesis. Although there may not be real changes in some apoptotic markers in our model, it is possible that there may be only limited numbers of cells that have undergone the apoptotic change and therefore differences in apoptotic regulatory molecules may be difficult to detect.

In addition to the more classic apoptotic regulatory genes mentioned above, there are other not well-characterized proteins that can exhibit antiapoptotic properties. CD44 is characteristic of intestinal stem cells and has been proposed to confer protection against apoptosis in colon cancer cells and in normal mouse intestine (13,22). It has been suggested that CD44 protects cells from Fas-mediated apoptosis (37) or through a shift in the BclXL:Bak ratio (13). In our model, CD44 expression increased in normal intestine and in tumors. We observed a resistance to apoptosis in the normal FD intestine and an increase in the BclXL:Bak ratio; this finding is consistent with the proposed antiapoptotic mechanism for CD44 in mouse colon (13).

Gsn is an actin regulatory protein that plays a role in cell motility, proliferation, and apoptosis (15,38). Although conflicting observations about its role in cancer have been reported (24,39,40), its antiapoptotic activity may be due to its ability to inhibit the loss of mitochondrial permeability and cytochrome c release (41) and to close the voltage-dependent anion channel in a similar manner to BclXL (42). Gsn also can inhibit caspases directly upon binding to phosphatidylinositol 4,5-bisphosphate (43). The increase in Gsn expression in normal intestine in our FD mice is consistent with the resistance to apoptosis and with the proposed antiapoptotic role of Gsn. Therefore, our data strengthen the argument for Gsn as an important apoptotic regulator.

Interestingly, although the Mthfr genotype increases tumor rate in this model, none of the apoptotic factors studied in this work were altered due to the Mthfr genotype. Therefore, apoptosis may not be associated with Mthfr-related tumor promotion and other mechanisms, such as modulation of DNA methylation, might be involved.

Folate deficiency in normal intestine was associated with an increase in CD44 and Gsn protein levels. Because we did not observe differences in the mRNA levels in these genes, there may be post-translational regulatory changes. On the other hand, as mentioned above, there may be limited numbers of cells that are
undergoing regulatory changes in the normal intestine at this time, particularly because the tumors are still quite small (0.8–1 mm) after 1 y of folate deficiency. Nonetheless, the involvement of CD44 and Gsn in tumor development in our model is quite clear based on the increased expression of both molecules at the level of mRNA and protein in the tumors. In summary, folate deficiency induces apoptosis resistance in normal preneoplastic intestine. This intriguing observation in an in vivo setting provides another important mechanism by which low folate can trigger tumor formation, particularly in the context of DNA damage. Increased expression of CD44 and Gsn and an alteration of the BclXL:Bak ratio contribute to the antia apoptotic effects and ultimately to the malignant transformation.

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Literature Cited


