Studies of methionine cycle intermediates (SAM, SAH), DNA methylation and the impact of folate deficiency on tumor numbers in Min mice

Sahar Sibani1, Stepan Melnyk2, Igor P.Pogribny2, Wei Wang1, Francois Hiou-Tim1, Liyuan Deng1, Jacquette Trasler1,2, S.Jill James2 and Rima Rozen1,4

1Departments of Pediatrics and Human Genetics, McGill University-Montreal Children’s Hospital Research Institute, 4060 Ste Catherine Street West, Montreal, Quebec H3Z 2Z3, Canada, 2Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, Arizona, USA and 3Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada

© Oxford University Press 61

Abbreviations: Min, multiple intestinal neoplasia; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

Several epidemiological studies have suggested a modulatory effect of dietary folate intake on the risk of colorectal cancer. The molecular basis for this inverse association is not clearly understood, but may involve alterations in DNA methylation. In this study, we examined the levels of methylation intermediates [S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH)] and of global DNA methylation in the pre-neoplastic small intestine of Min (multiple intestinal neoplasia) mice. We also studied the effect of folate/choline deficiency on these parameters and on tumor multiplicity in this animal model. In folate-deficient Min mice, we identified positive linear correlations between SAM or SAH and tumor numbers ($R^2 = 0.38$, $P < 0.005$; $R^2 = 0.26$, $P = 0.025$, respectively). A positive correlation between global DNA hypomethylation and tumor multiplicity was also observed ($R^2 = 0.29$, $P = 0.014$). These three biochemical determinants (SAM, SAH and DNA hypomethylation) may, therefore, serve as early markers of cell transformation. Folate/choline deficiency, however, did not produce a consistent effect on tumor numbers in three separate experiments. As an increase in tumor numbers was observed only in folate- and choline-deficient mice with low levels of SAM and DNA hypomethylation, the modulatory role of folate may be dependent on the transformation state of the cell.

Introduction

Colorectal cancer is the second deadliest cancer in the US with an estimated incidence of 0.5% for the year 2000 (1). Although several genetic alterations are involved in colorectal carcinogenesis, including mutations of the APC, K-Ras, DCC, MCC and p53 genes (2), it has been estimated that up to 90% of colorectal cancers can be avoided through dietary alterations (3). As a result, much effort is being expended in an attempt to identify natural and synthetic chemopreventive agents. Several epidemiological studies have suggested an inverse association between dietary folate intake and risk for colorectal adenomas and cancer (4–10). In these reports, individuals ingesting high amounts of folate (>0.4 mg daily) have approximately a 2-fold reduction in their risk compared with those with a daily intake of <0.2 mg.

Mechanisms of folate-related carcinogenesis are not completely understood. Folates are involved in several important single-carbon transfer reactions. 5,10-Methylenetetrahydrofolate is utilized for conversion of dUMP to dTMP, whereas formyltetrahydrofolate is required for purine synthesis. The reduced derivative of 5,10-methylenetetrahydrofolate, 5-methyltetrahydrofolate, provides the methyl donor for methionine and S-adenosylmethionine (SAM) synthesis; the latter is required for DNA synthesis (11). Consequently, folate deficiency could increase DNA damage (12,13) and reduce DNA repair efficiency due to perturbations in nucleotide synthesis (14–16). Alternatively, folate deficiency could mediate its effects by altering cellular methylation reactions. DNA methylation is an important epigenetic factor, inversely associated with gene expression (17,18). Abnormal methylation patterns have been detected early in cancers, including those of the colorectum (2,19,20). These changes consist mainly of global DNA hypomethylation, regional DNA hypermethylation and overexpression of DNA methyltransferase 1 (21).

Studies with the dimethylhydrazine-induced model of carcinogenesis in rats have suggested a cause-and-effect relationship, in which folate deficiency increased the incidence of tumors (22) whereas folate supplementation conferred protection (23). Recently, the effects of dietary folate intake were examined in the ApcMin+/Msh2−/− mouse (24). The APC gene is mutated in >80% of sporadic colorectal cancer cases (2), whereas the mismatch repair gene, MSH2, is altered in 21% of families with hereditary non-polyposis colorectal cancer (HNPPC) (25). In this mouse model, folate supplementation reduced the tumor multiplicity if administered before tumor development, whereas folate deficiency conferred protection after tumor formation (24). In a follow-up experiment (26), this group attempted to alter tumor multiplicity in the ApcMin+/Msh2−/− (Min) mouse using the same folate-deficient and -supplemented diets that had been effective in the ApcMin+/Msh2−/− mouse. No significant changes were observed when the whole small intestine (SI) was examined (26). However, when focusing on the different parts of the SI, they identified a significant linear decrease in the number of ileal adenomas with increasing dietary folate intake (26).

In this report, we examined the levels of methylation intermediates [SAM, S-adenosylhomocysteine (SAH)] and of global DNA methylation in pre-neoplastic flat small intestine of Min mice. We also evaluated the impact of these biochemical changes and of folate-deficient diets on tumor multiplicity.

Materials and methods

Biochemical measurements

Intracellular SAM and SAH levels were measured by high-pressure liquid chromatography with electrochemical detection (27). The samples from each...
experiment were all assayed at the same time to minimize between-group variation. All samples were repeated at least once with similar results.

Global DNA methylation was ascertained by the cytosine extension assay (28). Briefly, high-molecular-weight DNA was prepared using standard phenol–chloroform extraction methods, and 0.5 µg was digested with 5 U of the methylation-sensitive restriction enzyme HpaII (37°C, 4 h). A single nucleotide extension reaction was performed in 25 µl of reaction mixture containing the 0.5 µg of pre-digested DNA, 1× PCR buffer II, 1.0 mM MgCl2, 0.5 U AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) and 0.1 µl of [3H]dCTP (57.4 Ci/mmol, NEN, Boston, MA). The reaction mixture was incubated at 55°C for 1 h and then placed on ice to terminate the reaction. Undigested DNA (0.5 µg) from each sample served as a control for background incorporation. Aliquots (10 µl) from each reaction mixture were applied to Whatman DE-81 ion exchange filters. The filters were washed three times with 0.5 M Na–phosphate buffer (pH 7.0), dried in open air and counted in a scintillation counter. The level of DNA hypomethylation was calculated as the disintegrations per minute of HpaII-digested sample minus the disintegrations per minute of undigested sample. In this assay, the incorporation of [3H]dCTP is directly proportional to the number of unmethylated (cleaved) CpG sites in the original sample, and thus reflects the level of global DNA hypomethylation. All samples from experiments 1 and 2 were run at the same time to minimize variation.

Mice and dietary studies

Min mice, purchased from the Jackson Laboratory (Bar Harbor, Maine), were bred at the Montreal Children’s Hospital Research Institute’s animal care facility under a 12-h light:dark cycle. The mice were genotyped by a method described previously (26). Offspring of C57Bl/6J ApcMin/+ × C57Bl/6J matings were randomly assigned to either a control, or a folate- and choline-deficient (F0Ch−) diet at weaning, and continued to receive these diets until 13 weeks of age (91 days; composition of diets is shown in Table I). Choline deficiency was intended to exacerbate folate deficiency, as choline provides methyl groups that can be utilized in methionine synthesis. No antifolate drugs were added, as only a mild deficiency similar to that observed in the human population was desired. Mice were killed at 13 weeks of age. Tumor and pre-neoplastic small intestine tissue were removed, and snap frozen on dry ice. Pre-neoplastic mucosa was defined by the absence of visible adenomas under a dissecting microscope. The remaining small intestine was fixed in Bouin’s solution and examined for tumors under a dissecting microscope.

The experiments with control and deficient diets were repeated three times with three different litters from the C57Bl/6J ApcMin/+ × C57Bl/6J matings. In each of these experiments, folate deficiency did not significantly affect the body weight of animals (data not shown).

Results

SAM, SAH and global DNA methylation in pre-neoplastic small intestine of Min mice

Previous studies have reported changes in SAM, SAH and DNA methylation during tumor progression. We examined these three parameters in the pre-neoplastic cells of the flat small intestine in Min mice fed the control diet. Figure 1 shows the combined results from the three different experiments. A positive linear correlation for SAM with tumor multiplicity was identified (Figure 1A; $R^2 = 0.38, P \leq 0.005$). A similar observation was made for SAH (Figure 1B; $R^2 = 0.26, P = 0.025$). A linear correlation was also found between DNA hypomethylation and tumor multiplicity (Figure 1C; $R^2 = 0.29, P = 0.014$). Thus, an increase in SAM, SAH and DNA hypomethylation appeared to correlate with increased tumor multiplicity in the folate-adequate Min mice.

Dietary folate deficiency and tumor multiplicity

Human epidemiological data have suggested an increased risk for colorectal cancer with a low dietary intake of folate. We therefore examined tumor multiplicity in Min mice fed a folate- and choline-deficient diet (F0Ch−). The effect of folate deficiency was not consistent between the three experiments replicated in three different sets of mice (Figure 2). In the first experiment, there was a 2-fold significant increase in tumor numbers, whereas in the second and third experiments there were statistically non-significant decreases of 34 and 46%, respectively.
SAM, SAH, DNA methylation and folate deficiency

Biochemical changes associated with dietary-induced folate deficiency

As folate-derived methyl groups contribute directly to SAM synthesis, we examined the levels of SAM in the flat small intestine of the parental non-mutant C57Bl strain to ensure that our F0Ch diet was effective in reducing SAM levels. A clear decrease in SAM was observed in the flat small intestine (mean of 4 ± SE = 0.21 ± 0.03 nmol/mg protein for the control diet; the values for the two mice in the deficient group were 0.07 and 0.052). The SAH values were not substantially affected by diet (mean of 4 ± SE = 0.07 ± 0.005 nmol/mg protein for the control diet; the values for the two F0Ch mice were 0.044 and 0.059). Global DNA methylation was similar in the two groups (mean of 4 ± SE = 1343 ± 39 d.p.m.; the values for the two mice in the deficient group were 1763 and 1343). The folate-deficient diet was clearly effective in reducing SAM levels in the background control strain.

As the Min mice of experiments 2 and 3 both displayed non-significant decreases in tumor multiplicity in response to the F0Ch diet, whereas the mice in experiment 1 showed an increase in tumor number, we decided to compare the mice used in experiments 1 and 2 for changes in levels of SAM, SAH and DNA hypomethylation. In experiment 1, the pre-neoplastic small intestine showed a significant decrease in SAM accompanied by a significant increase in DNA hypomethylation (Figure 3A and C, respectively). No significant change in SAH was observed (Figure 3B). In experiment 2, no significant changes in SAM, SAH or DNA hypomethylation were observed between folate-adequate or -deficient mice nor was there a significant difference in tumor number between groups. However, a comparison of the control mice between experiments 1 and 2 revealed an increase in DNA hypomethylation that was associated with increased tumor multiplicity (Figures 2 and 3C).

Discussion

In humans, dietary folate intake has been inversely correlated with the risk for colorectal adenomas and cancer. The main biochemical role of folate is the transfer of one-carbon moieties (29). In that respect, folate is an important factor in DNA synthesis/repair, and in methylation, as it provides methyl groups for synthesis of thymidine/purines and SAM, respectively (30). Several studies have identified abnormal DNA methylation patterns in neoplastic tissue (21). However, very few reports have examined pre-neoplastic cells. Results from our study indicate that in the folate-adequate Min mice, the level of global DNA hypomethylation in the pre-neoplastic small intestine is positively correlated to tumor multiplicity. This is consistent with reports of decreased DNA methylation in neoplastic cells (21) and may reflect initiation of tumorigenesis in a greater number of small intestinal cells in mice with hypomethylated DNA. Thus, the greater the number of cells that have initiated tumorigenesis, the more tumors the animal is likely to develop.

Alterations in DNA methylation could be caused by several potential factors, including changes in the concentration of substrate for the methyltransferase, SAM, and/or in its inhibitor, SAH. Our results show that mice with more hypomethylated intestinal DNA, and thus a greater tumor multiplicity, have elevated levels of both SAM and SAH (Figure 1A and B).
Experimentally induced increases in SAH have confirmed its role as a potent product inhibitor of DNA methyltransferase (31). In several studies, methyltransferase inhibition was accompanied by increases in SAM, presumably due to decreased utilization. These observations provide a possible explanation for the parallel increases in SAM and SAH associated with DNA hypomethylation and increased tumorigenicity in the folate-adequate Min mice. The flat small intestines of Min mice have higher levels of polyamines than those of C57Bl mice (32). Polyamines are required for cell growth and proliferation, and are synthesized from decarboxylated SAM. Thus, increased production of SAM may be required to maintain the cell’s demand for greater polyamine synthesis. This is partially supported by the recent finding that methionine adenosyltransferase II (MAT II), the enzyme required for SAM synthesis in non-hepatic cells, is upregulated in colon cancer cells (33), indicating a possible increase in SAM synthesis. Thus, the observed increase in SAM in the pre-neoplastic small intestine of Min mice may represent the increased synthesis and/or reduced utilization of SAM. Taken together, our findings suggest that increases in SAM, SAH and DNA hypomethylation may be considered as early markers of tumorigenesis in the folate-adequate Min mice.

The folate-deficient diet did not produce consistent changes in tumor numbers in three experiments using mice from three different C57Bl/6JApCMin/+ × C57Bl/6J matings. The inconsistent observations may be attributable to subtle differences in the pre-neoplastic state of the mucosal cells between the different litters at the time the F0Ch− diet was administered. In the very early stages of tumorigenesis, in which the cells are expected to have normal levels of DNA methylation, the folate-deficient diet may be tumor promoting. This possibility is consistent with the results of experiment 1 in which the folate-deficient diet resulted in DNA hypomethylation and an increase in tumor multiplicity. On the other hand, in cells at a later stage of tumor progression, the folate-deficient diet may provide a protective effect by decreasing proliferation and increasing apoptosis (34). This interpretation is consistent with our results from experiment 2, where the F0Ch− diet decreased tumor number, although the effect was statistically non-significant. This interpretation is also consistent with the results reported by Song et al. (24) in which a folate-deficient diet initiated after the establishment of neoplastic foci decreased tumor multiplicity in the ApcMin/+ Msh2−/− mice.

The C57Bl/6JApCMin/+ mice are murine models of human familial adenomatous polyposis (FAP), a hereditary early-onset colon cancer with a germline mutation in the APC gene. The majority of sporadic colon cancers also carry mutations in the APC gene, although this initiator mutation is presumably acquired later in life. The FAP patients and Min mice could be viewed as having accelerated forms of cancer, due to the presence of the APC mutation at birth (with acquisition of a second mutation at a later time). Despite subtle differences between various murine models and human colon cancer, Min mice have been successfully used to test and identify chemopreventive agents against colon cancer, such as non-steroidal anti-inflammatory drugs (35,36). However, their accelerated rate of initiation of intestinal adenomas may impose limitations on the types of studies in which they can be useful. With respect to folate deficiency, our work suggests that the timing of the deficiency, relative to the initiation state, may determine whether the deficiency will promote or inhibit tumorigenesis.

In conclusion, three early indicators of tumor multiplicity in the Min mouse were identified: SAM, SAH and DNA hypomethylation. Both SAM and SAH displayed positive correlations with tumor multiplicity, as did DNA hypomethylation. The folate-deficient diet did not demonstrate a consistent effect on tumor multiplicity between the three separate experiments. This may reflect differences between experiments in the baseline state of transformation in the different cell populations. Folate deficiency may promote tumorigenesis in cells that are in the earliest stages of tumor progression, while inhibiting tumor progression in cells that are more advanced in tumor progression. Measurements of the various folate derivatives and other relevant indicators such as the activity of the DNA methyltransferase may provide clues as to whether folate deficiency will be tumor promoting or tumor protective in this model.

Acknowledgements
This work was supported by the Cancer Research Society of Canada and the Canadian Genetic Diseases Network. R.R. is a Senior Scientist of the Canadian Institutes for Health Research (CIHR). J.T. is a Scientist of the CIHR and a Scholar of the Fonds de la recherche en santé du Québec.

References


Received June 12, 2001; revised September 27, 2001; accepted October 9, 2001