Dietary Folate Deficiency Blocks Prostate Cancer Progression in the TRAMP Model

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Abstract

Dietary folate is essential in all tissues to maintain several metabolite pools and cellular proliferation. Prostate cells, due to specific metabolic characteristics, have increased folate demand to support proliferation and prevent genetic and epigenetic damage. Although several studies have found that dietary folate interventions can affect colon cancer biology in rodent models, its impact on prostate is unknown. The purpose of this study was to determine whether dietary folate manipulation, possibly being of primary importance for prostate epithelial cell metabolism, could significantly affect prostate cancer progression. Strikingly, mild dietary folate depletion arrested prostate cancer progression in 25 of 26 transgenic adenoma of the mouse prostate (TRAMP) mice, in which tumorigenesis is prostate-specific and characteristically aggressive. The significant effect on prostate cancer growth was characterized by size, grade, proliferation, and apoptosis analyses. Folate supplementation had a mild, nonsignificant, beneficial effect on grade. In addition, characterization of folate pools (correlated with serum), metabolite pools (polyamines and nucleotides), genetic and epigenetic damage, and expression of key biosynthetic enzymes in prostate tissue revealed interesting correlations with tumor progression. These findings indicate that prostate cancer is highly sensitive to folate manipulation and suggest that antifolates, paired with current therapeutic strategies, might significantly improve treatment of prostate cancer, the most commonly diagnosed cancer in American men. Cancer Prev Res; 4(11); 1825–34. ©2011 AACR.

Introduction

Folate (vitamin B9) is essential to sustain proliferation in all cell types because it is part of a complex metabolism that ultimately provides de novo dTTP and S-adenosylmethionine (AdoMet; Fig. 1). In turn, dTTP is necessary for DNA synthesis whereas AdoMet is the universal intracellular methyl donor for methylases including DNA and histone methylases. For this reason, antifolate therapy has been used for decades to treat certain types of cancer (1). Conversely, folate supplementation, by supporting the maintenance of these pools, might prevent genetic and epigenetic damage and the consequent development of neoplasia.

Importantly, AdoMet is also used, through decarboxylation, for the biosynthesis of polyamines (refs. 2, 3; Fig. 1). We have previously shown that heightened polyamine biosynthesis, consequent to secretory function of prostate epithelial cells, draws on AdoMet pools (Fig. 1) and makes prostate cancer cell growth in vitro extremely dependent on folate (4). Nonetheless, the effects of dietary manipulation of folate on the prostate in vivo are currently unknown.

Folate fortification of foods became mandatory in the United States in 1998 to benefit pregnant women and prevent neural tube defects in newborns. However, folate supplementation might be detrimental for men at risk for prostate cancer, as suggested by the results of a recent clinical trial in which supplementation resulted in a significant increase in the incidence of prostate cancer (5, 6). Indeed, dietary supplementation of folate has been referred to as a "double edged sword" (7), as epidemiologic studies over the past decade suggest an inverse association of dietary folate intake with the risk of several malignancies, including colon and liver cancer (8, 9), yet supplementation could also sustain and accelerate the growth of pre-existing cancer cells (7, 8).

Given that prostate cancer is the most commonly diagnosed and second most lethal cancer in men in the United States (10), and in light of our findings that prostate metabolism increases demand on folate intake (4, 11), there is a pressing need to have a better understanding of...
how dietary folate influences prostate cancer in vivo. We therefore studied the effects of dietary folate manipulation in the transgenic adenoma of mouse prostate (TRAMP) model, where tumorigenesis is driven by androgen-dependent expression of the SV40 large and small T antigen specifically in the prostate at puberty (12). The results indicated that dietary folate restriction, at a level sufficient to sustain normal mouse growth, dramatically and significantly blocked prostate cancer progression in TRAMP mice, in which tumorigenesis is considered to be very aggressive. Conversely, dietary folate supplementation had no significant effect on prostate cancer growth or disease progression, though there is some suggestion of moderation of the phenotype. Further investigation into mechanisms associated with growth arrest and recovery consequent to folate deficiency suggested that upregulation of the enzymes involved in dTTP biosynthesis [thymidylate synthase (TS)] and salvage [thymidylate kinase (TK)] may be important.

Our study suggests that antifolate treatment and/or specific inhibitors aimed at counteracting upregulation of key biosynthetic/salvage enzymes could be a new promising therapeutic approach for prostate cancer.

Materials and Methods

Mice and dietary intervention

All the mouse work was carried out at the Department of Laboratory Animal Research at Roswell Park Cancer Institute. Male TRAMP mice (12), heterozygous for the Pb-Tag transgene [(C57BL/6J × FVB)F1 background], were bred in the Roswell Park Cancer Institute animal housing facility in accordance with an Institutional Animal Care and Use Committee—approved protocol. Mice were weaned at 3 weeks of age. At the time of weaning, the mice were randomly assigned to 3 cohorts characterized by different folate concentrations in their diet until they reached 22 weeks of age, at which time the mice were sacrificed. The same was applied to 3 cohorts of mice carried out only till 12 weeks of age but with larger cohort size due to the small size of the prostate at this age (~25 mg for lateral, dorsal, and ventral lobe combined) to ensure enough prostatic tissue to carry out the presented analyses. Each cohort at both time points had at least 25 mice. The time points of 12 and 22 weeks were selected to assess a possible effect of the diets on both early (12 weeks) and late (22 weeks) prostate tumorigenesis. A priori power analysis determined that with a cohort size of 20, we would have 80% power to detect an effect size of 0.96 at a significance level of 0.05, using the Wilcoxon rank-sum test to determine whether the diets affect various aspects of disease progression. Starting with a cohort size of 25, we achieved a minimum of 20 samples per diet for most measurements.

The 3 amino acid–defined diets were as follows: (i) a folate control diet containing the recommended amount of folic acid for rodents (2 mg/kg diet; refs. 13, 14), (ii) a folate-deficient diet (0.3 mg/kg diet; refs. 14, 15), and (iii) a folate-supplemented diet (20 mg/kg diet), all supplemented with 1% succinylsulfathiozole to inhibit folic acid synthesis by intestinal flora, and all containing 3.3 mg/kg of methionine, 2.5 g/kg choline with energy provided as 15.5% l-amino acids, 66% carbohydrates, and 18.5% fat (14, 15). All 3 diets are identical with the exception of the amount of folic acid. The diets were purchased from Harlan Teklad, stored at 4°C, and fed without restriction with replacement every second day. The diets were TD.01369 (control), TD.01546 (folate deficient), and TD.08149 (folate supplemented). Urogenital tract (UG) weight and body weight were determined at sacrificing. Tissue samples were obtained as previously described (16).

Tissue and serum folate measurements

Serum and tissue folate levels were quantified using the Lactobacillus casei microbiological assay as previously described (4, 17). Tissue folates were digested with recombinant rat conjugase [a kind gift of Karen Chave, research
scientist at Wadsworth Center, New York State Department of Health (NYSDOH) before analysis with *L. casei*. Complete digestion of poly-γ-glutamyl folates into the monoglutamate form (folic acid) by the conjugase was verified by comparing standard curves generated with folic acid and pteroylheptaglutamate (PteGlu7, a kind gift of Dr. John McGuire, Roswell Park Cancer Institute, Buffalo, NY) before and after digestion.

**Histopathology**

Histopathology of tissues of TRAMP mice was carried out as previously described (18). The dorsal, lateral, and ventral prostates lobes were scored for tumor grade by hematoxylin and eosin (H&E) in mice on all 3 diets at both 12 and 22 weeks of age. The previously established scoring system in which the prostate lobes are graded 1 to 6 was used as follows: 1, normal mouse prostate; 2, low-grade prostatic intraepithelial neoplasia (PIN); 3, high-grade PIN; 4, well-differentiated adenocarcinoma; 5, moderately differentiated adenocarcinoma; and 6, poorly differentiated adenocarcinoma with sheets of anaplastic cells (18). Disease index for each mouse at 22 weeks was calculated by averaging the predominant H&E grade for each lobe plus the average worst grade for each lobe multiplied by the UG weight (19). A total of 25 slides were analyzed per diet per time point for immunohistochemical (IHC) staining of Ki-67 and caspase-3. Analyses were carried out on the entire section with the percentage of positively stained cells counted manually and each section categorized as having <5%, 5%–25%, 26%–50%, 51%–75%, or >75% positive cells, as shown in Figure 3B. Analyses were carried out blind to both diet and age. Statistical analysis was carried out using a 2-tailed Fischer’s exact test for the comparisons described.

**High-performance liquid chromatography analyses.** High-performance liquid chromatography analyses for polyamine, nucleotide, and AdoMet pools measurements were carried out as previously described (11, 20–22). All analyses were carried out on a reverse-phase Econosil (C18) column (5-μm particle size; 4.6 × 250 mm; Fisher Scientific) with a C18 guard column assembled on the Waters 2796 Bioseparation module of the Biopolymer Facility, at Roswell Park Cancer Institute (Buffalo, NY).

**Quantitative reverse transcriptase PCR.** One microgram of RNA was retrotranscribed with the Single-Strand cDNA Synthesis Kit (Fermentas) in a 20 μL reaction solution using random hexamers. One microliter of the so-obtained cDNA was used for real-time reverse transcriptase PCR analyses, in triplicate, with the iTaq SYBR Green supermix with ROX (Bio-Rad) on an ABI 7900HT (Applied Biosystems). Primers for TS, TK, and 18S are shown in Supplementary Table S1.

**Random oligonucleotide-primed synthesis.** Random oligonucleotide-primed synthesis (ROPS) was carried out as previously described (11, 23). Briefly, DNA was denatured and rapidly cooled down, causing single-strand DNA breaks to provide random priming for successive DNA synthesis with labeled nucleotides. Radioactivity incorporation provides a reproducible, quantitative assessment of single-strand DNA breaks. Pretreating DNA with uracil glycosidase and Exo III allows quantification of uracil misincorporation. At least 5 independent replicates per sample were analyzed. Uracil misincorporation was normalized, as recommended, by subtracting the background due to preexisting single-strand breaks and apyrimidin sites. The so-obtained data expressed as dCTP incorporated per microgram of DNA (dCTP/μg).

**Mass array quantitative methylation analysis by Sequenom.** Briefly, the DNA was bisulfite treated (EZ DNA methylation kit; Zymo Research) and PCR amplified (HotStarTaq DNA Polymerase; Qiagen) with bisulfite sequencing primers flanking the CpGs of interest at loci identified by restriction landmark genomic scanning. Mass array quantitative methylation analysis (MAQMA) was carried out using the MassARRAY Compact System developed by the Sequenom company, as previously described by Ehrich and colleagues (24). This system uses matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry for the detection and quantitative analysis of DNA methylation. This approach has been shown to be a highly accurate and reproducible way to quantitate DNA methylation (25). Primers for the MAQMA assays are shown in Supplementary Table S1.

**Results**

**Dietary folate status affects serum and tissue folate levels and disease progression.** TRAMP mice were placed on the experimental diets immediately after weaning (3 weeks old) and kept on the diets until either 12 or 22 weeks. The diets had no significant effect on body weight (Supplementary Fig. S1B) yet significantly affected serum and prostate folate levels at both 12 and 22 weeks and liver folates at 22 weeks (Supplementary Fig. S1). Tissue folate levels significantly correlated with serum levels as shown in Figure 2A.

To assess how the dietary interventions affected the course of disease in this model, we measured the UG weight normalized to body weight (UG/body weight) at 12 (n = 28/diet; not shown) and 22 weeks (Fig. 2B). At 12 weeks, the distribution of UG/body weights was equal across the 3 diets (not shown). At 22 weeks, however, the data were striking. The distribution of UG/body weights in the control group exhibited significant heterogeneity. This distribution into 2 distinct populations (mice that developed large tumors vs. mice that did not) is typical of this model (18). There was no difference in distribution for mice fed the folate-supplemented diet. Mice fed the deficient diet, however, showed a remarkable reduction (P < 0.01) and uniformity in UG/body weights, with the exception of a single animal in which the tumor escaped the growth-suppressive effects of low dietary folate (Fig. 2B). Ten of 24 animals had a normalized UG weight greater than 50 mg when fed the control diet as compared with only 1 of 26 in mice fed the folate-deficient diet. These data suggest that the folate-deficient diet caused a dramatic block in tumor growth whereas folate supplementation had little or no effect on overall tumor growth.
Folate deficiency improves prostate cancer pathology in TRAMP. Histologic examination of prostate tissue of TRAMP mice revealed additional effects of the dietary manipulation. The dorsal, lateral, and ventral prostate lobes were scored for tumor grade by H&E in mice on all 3 diets at both 12 and 22 weeks of age as described in Materials and Methods. Representative H&E sections from prostate lobes at 22 weeks are shown in Figure 3. Figure 4A shows the number of cases scored as each grade for ventral prostate at 22 weeks. Prostate lobes at 12 weeks were nearly all characterized by early lesions of intraepithelial neoplasia, with no significant difference among the diets (Supplementary Fig. S2). Strikingly, at 22 weeks on the folate-deficient diet, there was little or no progression from what was observed at 12 weeks, with the majority of prostates still scoring as grade 3 and no grade 6 tumors. In the control and supplemented groups, however, there were 10 and 7 grade 6 tumors, respectively (Fig. 4A). Thus, dietary folate deficiency resulted in a significant reduction in mice that progressed beyond grade 3, high-grade PIN ($P = 0.02$).

Neither diet affected transgene or androgen receptor expression (not shown), with all mice showing a very high percentage of strongly staining cells specifically in the prostate. No evidence of distant micrometastatic spread to the liver or kidney was found in any animal. However, folate deficiency significantly reduced spread to the peri-aortic lymph node ($P = 0.05$). Lymph node metastases were assessed by both H&E and IHC staining using antibody to the transgene, as exemplified in Supplementary Figure S3. Lymph node metastases were found in 10 of 20 mice on the control diet compared with 4 of 21 in mice on the deficient diet and 8 of 18 mice on the supplemented diet (Fig. 4E). To quantify overall UG disease for each animal, a disease index was derived (19). Folate deficiency significantly reduced the disease index with only 1 of 21 animals having a disease index greater than 25 compared with 9 of 22 animals on the control diet ($P = 0.01$). Folate supplementation had a slight, yet nonsignificant ($P = 0.1$) moderating effect with only 3 of 19 animals having a disease index greater than 25 compared with 9 of 22 in the control diet. There were no differences in disease index among the 3 diets at 12 weeks of age (not shown).

Proliferative and apoptotic indices, as measured by Ki-67 and cleaved caspase-3 staining, respectively, showed that...
prostates of mice fed the deficient diet were characterized by a significantly lower number of proliferating cells (Supplementary Fig. S4 and Fig. 4B; \( P = 0.02 \)) and apoptotic cells (Supplementary Fig. S4 and Fig. 4C; \( P = 0.05 \)) whereas folate supplementation had no effect. Cell surface E-cadherin staining, a marker of differentiation that is often lost during the process of malignant progression (26), was significantly retained in mice on the deficient diet compared with mice fed the control or supplemented diets (Fig. 4D; \( P = 0.02 \)). We conclude that although folate supplementation had no significant effect on prostate tumorigenesis in TRAMP mice, the folate-deficient diet
effectively prevented prostate cancer growth by blocking proliferation, lymph node metastasis, and acquisition of a poorly differentiated phenotype but not by increasing apoptosis.

**Genomic effects of dietary folate manipulation in TRAMP.** Folate depletion hinders cell proliferation by decreasing the availability of AdoMet and dTTP, yet it might induce genetic and epigenetic damage and worsen the phenotype of cells that are able to proliferate. Specifically, folate deficiency limits the ability of the cells to convert dUMP to dTMP (Fig. 1) and ultimately results in an increased dUTP:dTTP ratio (U:T), which, in turn, may lead to futile cycles of uracil misincorporation into the DNA followed by its excision and consequent single-strand DNA breaks (11, 27–30). Similarly, decreased AdoMet pools may affect DNA and histone methylation.

Neither AdoMet nor S-adenosylhomocysteine (AdoHcy) pools were affected by the diets in the diseased prostate (not shown). However, CpG island hypermethylation was dramatically prevented by the folate-depleted diet. CpG island methylation status was determined by MAQMA (Fig. 5A) at 4 CpG islands previously found to be commonly methylated in TRAMP tumors (31). The data in Figure 5A show that these 4 CpG islands were not hypermethylated in mice on the folate-deficient diet but were hypermethylated on the control diet. For all 4 CpG islands, there was one clear exception in the folate-deficient group; the one animal that developed a large tumor. As was found about disease index (Fig. 2C), folate supplementation moderated the phenotype. For all 4 CpG islands, there were fewer tumors with high-level methylation in the supplemented group than in the control group.

Measurement of nucleotide pools (Fig. 5B) and genetic damage (Fig. 5C) revealed a significant increase in dUTP but no change in uracil misincorporation into the DNA or

**Figure 5.** Genetic and epigenetic damage assessment in prostate lobes of TRAMP mice. A, MAQMA of 4 loci frequently methylated during tumor progression in the TRAMP model. The average level of methylation detected by MAQMA across the sequenced fragment of the CpG islands for each samples is shown on the y-axis. This value comes from taking the average of MAQMA values for each CpG dinucleotide sequenced for each sample. Each symbol represents a single sample. B, HPLC analysis of dTTP, dUTP, and the dUTP:dTTP ratio in 22-week-old TRAMP mice fed the folate-defined diets. C, ROPS analysis of prostate tissue in 22-week-old TRAMP mice on the folate-defined diets quantifies single-strand DNA breaks before and after the removal of misincorporated uracil. Single-strand DNA breaks are directly proportional to the amount of radioactive dCTP incorporated (y-axis). Statistical comparisons were made between the control diet and each of the experimental diets individually, using a 2-tailed Mann–Whitney U test. Statistical significance is indicated as follows: *, *P < 0.05; **, *P < 0.01; and ***, *P < 0.001. Def, deficient; Ctrl, control; Sup, supplemented.
single-strand breaks in the prostates of mice on the folate-deficient diet at 22 weeks. We found no significant change in uracil misincorporation and nucleotide pools at 12 weeks (data not shown). Interestingly, the prostate of the one TRAMP mouse that developed a large tumor on the deficient diet (Fig. 2C) had the highest levels of both uracil misincorporation and single-strand breaks as compared with any other mouse on this diet (Fig. 5C; solid symbol). In contrast, significant changes in nucleotide pools were detected in the liver (Supplementary Fig. S5), confirming previous reports and suggesting that the response of prostate to dietary manipulation of folate might indeed be different.

We assessed the mRNA levels of the enzymes involved in dTTP biosynthesis (TS) and salvage (TK; Fig. 1) in the prostates of 22-week-old TRAMP mice. Both TS and TK levels significantly correlated with UG/body weight (Fig. 6A). TS expression was not significantly affected by either diet, but TK expression was significantly reduced in mice on the folate-deficient and folate-supplemented diets (Fig. 6B). Interestingly, the only prostate overexpressing both enzymes in the folate-deficient group was from the one mouse that overcame the growth-suppressive effects of low dietary folate and developed a large tumor (Fig. 6B; solid symbol).

Discussion

This study addresses how dietary folate manipulation impacts prostate cancer progression in an initiated mouse model. Our previous findings linking polyamine production to increased demand on folate intake to sustain prostate cancer cell proliferation (4) and maintain the genome (11) provided a compelling rationale to expect that prostate cancer would be extremely sensitive to availability of folate. Indeed, we found that mild dietary folate deficiency strongly blocked disease progression in TRAMP despite continued expression of the SV40 large T antigen and androgen receptor. Lack of polyamine production did not explain the lack of tumor growth on the deficient diet, as polyamine pools were not reduced by the diet (Supplementary Fig. S6). Indeed, polyamine levels are strictly maintained intracellularly, as shown by the large body of publications that in the past 20 years reported that targeting of polyamine biosynthesis to arrest cancer growth is largely ineffective due to complex compensatory mechanisms (2, 32). High levels of polyamine synthesis required by the prostate are indeed maintained under conditions of low folate, at the expense of other critical pathways. Specifically, folate depletion in our model led to growth arrest of prostate cells, even under the strong proliferation drive of the antigen (SV40-Tag).
Folate supplementation did not accelerate tumor growth or disease progression, despite a demonstrable increase in prostate tissue folate. However, the supplemented diet did significantly reduce the numbers of mice with aberrant methylation of 3 of 4 CpG islands tested (Fig. 5), suggesting that there was some effect on the disease process. In addition, folate supplementation significantly reduced the expression of TK, the key regulator of the salvage pathway for maintaining dTTP pools (Fig. 6). Furthermore, supplementation seems to have reduced the overall disease index for the TRAMP mice (Fig. 2C). The lack of statistical significance ($P = 0.1$) is likely driven by the large amount of heterogeneity of the TRAMP model on the control diet, as the observed variance was larger than expected (Fig. 2B and C). These data suggest that folate supplementation may have some beneficial effects on prostate cancer progression in this very aggressive model. However, the aggressiveness and heterogeneity of the TRAMP model may have overwhelmed the potential for folate supplementation to improve or worsen tumor progression and/or limited our ability to observe such changes without doubling the sample size. Further studies with larger sample sizes using different models of prostate cancer will be necessary to elucidate this point.

The effect of dietary deficiency of folate on prostate cancer progression in the TRAMP model (blocking progression) is particularly striking because this same diet initiated intestinal tumors when fed to wild-type mice for 12 months (14) and increased adenoma number in Apc$^{min/+}$ mice (15). A key relevant difference between prostate tissue and colon tissue might be polyamine biosynthesis. Prostate cells are characterized by an unusually high rate of polyamine biosynthesis (33–36), which is maintained even in the face of low levels of intracellular folates (Supplementary Fig. S6). This renders prostate exquisitely sensitive to folate depletion, because a significant amount of 1-carbon units has to be devoted to maintain the high polyamine biosynthesis characteristic of this organ (4, 11). Their acetylation and consequent secretion due to the activity of spermidine/spermine N1-acetyltransferase (SSAT) draws on AdoMet pools to maintain intracellular polyamine levels (37). This leads to increased demand for folate input to maintain AdoMet pools, polyamines, and proliferation (4). When transgenic mice overexpressing SSAT were crossed with Apc$^{min/+}$ mice, the result was a 6-fold increase in colon adenoma number (38). However, when crossed with the TRAMP mouse, the result was the opposite—greatly reduced prostate tumor growth (19). This was likely due to acetyl Co-A pool depletion, as suggested by the authors, and perhaps also due to metabolic stress on the methionine cycle and 1-carbon metabolism because of enhanced flux to maintain intracellular polyamine pools. Although there are caveats to these comparisons due to the different genetic backgrounds and transgenes, the findings nevertheless suggest that differences in metabolic processes between colon and prostate tissue might significantly impact the biological consequences of their manipulation.

Antifolate cancer therapy has been widely used in the past 30 years to treat various malignancies, but the effects of antifolates on primary prostate cancer are unknown. Positive results for treating castration recurrent prostate cancer with methotrexate (39, 40) were not confirmed by later studies (41–43). We suggest that the high level of polyamine biosynthesis due to the secretory characteristics of prostate uniquely stresses 1-carbon metabolism and the methionine cycle and is therefore central to the rationale behind applying antifolate therapy to prostate cancer. The enzymes responsible for synthesizing and exporting polyamines are regulated by androgens, which keep their expression levels high in prostate tissue (35, 44–46). Although it is known that intracellular polyamine concentration increases upon tumor progression, the effects of androgen deprivation therapy on these enzymes and therefore overall prostate polyamine synthesis are unknown and might in fact strongly weaken the potential of antifolates in the castration recurrent setting. Therefore, its failure in this advanced setting should not predict failure at earlier time points where polyamine biosynthesis is known to be high and driven by androgens.

Our findings are significant from both a dietary recommendation perspective and a therapeutic perspective. Folate supplementation, as well as fortification of certain foods, has the potential to protect from cancer development but may also accelerate the growth of neoplasms that may already be present, though subclinical (7, 47). However, the rate of indolent prostate cancer in men older than 50 years is high (48), and it is possible that folate supplementation in these men might in fact accelerate prostate cancer growth. Conversely, our findings suggest that prostate cancer, due to its high dependence on folate, might be sensitive to antifolate-based approaches, perhaps not in the context of castration recurrence and chemotherapy resistance but rather in the context of earlier treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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