The Effect of Folic Acid Deficiency and MTHFR C677T Polymorphism on Chromosome Damage in Human Lymphocytes in Vitro

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Abstract

We performed a comprehensive study on the genotoxic and cytotoxic effects of in vitro folic acid deficiency on primary human lymphocytes. Lymphocytes were cultured in medium containing 12–120 nM folic acid for 9 days in a novel cytokinesis-block micronucleus (CBMN) assay system (n = 20). Besides identifying optimal folic acid concentrations for in vitro genomic stability, we tested the hypothesis that lymphocytes from individuals homozygous for the C677T methylenetetrahydrofolate reductase (MTHFR) polymorphism (TTs, n = 10) are protected against chromosome damage relative to controls (CCs, n = 10) under conditions of folic acid deficiency. This hypothesis is based on the assumption that reduced MTHFR activity in TT lymphocytes causes a diversion of 5,10-methylene tetrahydrofolate toward thymidine synthesis, which minimizes uracil-induced double-stranded DNA breakage. Cells were scored for micronuclei, apoptosis, necrosis, nucleoplasmic bridges, and nuclear budding. The latter two endpoints are indicative of chromosome rearrangements and gene amplification, respectively, and to the best of our knowledge, this is the first report of their association with folic acid concentration. Folic acid concentration correlated significantly (P < 0.0001) and negatively (r = −0.63 to −0.74) with all markers of chromosome damage, which were minimized at 60–120 nM folic acid, much greater than concentrations assumed “normal,” but not necessarily optimal in plasma. Two-way ANOVA revealed no effect of the MTHFR genotype on any of the endpoints. Results show that the C677T polymorphism does not affect the ability of a cell to resist chromosome damage induced by folic acid deficiency in this in vitro system.

Introduction

Cancer is one of several diseases that have been associated with low blood concentrations of folate (1). Folate provides methyl groups for DNA methylation and the conversion of uracil (dUMP) to thymidine (dTMP). Therefore, folate deficiency can disrupt global DNA methylation patterns (2) and induce excessive incorporation of uracil into DNA (3, 4). Simultaneous removal of two uracil bases on opposite DNA strands within 12 bp may result in the formation of DSBs (5). Uracil incorporation into DNA and subsequent chromosome breakage caused by DSBs is important because it is now established that the accumulation of chromosome aberrations is a risk factor for cancer (6, 7) and may be one of the underlying mechanisms of aging (8).

Studies have shown that global DNA hypomethylation, a marker of folic acid depletion, may induce chromosome loss, probably because of the undercondensation of pericentromeric heterochromatin (9). Chromosome loss leads to micronuclear formation and aneuploidy, the latter now recognized as a potentially important risk factor for cancer (10).

Perhaps one of the most intriguing facets of the relationship between folate status and cancer risk is how polymorphisms in key folate-metabolizing enzymes can modulate cancer risk. For example, the common C677T transition in the gene encoding MTHFR is reported to significantly reduce the risk for colorectal carcinoma (11–13) and acute lymphoblastic leukemia (14). MTHFR catalyzes the conversion of 5,10-MnTHF to 5-MeTHF. This latter folate species is the methyl group donor for the remethylation of homocysteine to methionine (see Fig. 1). Individuals who are heterozygous (CTs) and homozygous (TTs) for this MTHFR polymorphism have an in vitro enzyme activity that is 65 and 30% of normal, respectively. Elevated plasma homocysteine concentrations in TTs also indicates a reduced in vivo MTHFR activity (15–17).

It has been hypothesized that TTs are protected against some cancers because reduced MTHFR activity causes a diversion of 5,10-MnTHF toward thymidine synthesis (3). This altered folate distribution is thought to minimize intracellular dUMP:dTMP ratios and, therefore, the chance of uracil being incorporated into DNA. This report presents an in-depth comparison of markers of chromosome damage in lymphocytes using the CBMN assay between CCs and TTs during in vitro folic acid depletion and repletion. The objectives of this study

2 The abbreviations used are: DSB, double-stranded (DNA) break; BNF, binucleated (cells); Budd, nuclear bud; CBMN, cytokinesis-block micronucleus (assay); CC, persons nullizygous for the MTHFR C677T polymorphism; TT, persons homozygous for the MTHFR C677T polymorphism; MMN, micronucleated (cells); MN, micronuclei; 5,10-MnTHF, 5,10-methylene tetrahydrofolate; 5-MeTHF, 5-methyl tetrahydrofolate; MONO, mononucleated (cells); MTHFR, methylene tetrahydrofolate reductase; ND1, nuclear division index; NPB, nucleoplasmic bridge; PHA, phytohemagglutinin; BFF, breakage-fusion-bridge; CSIRO, Commonwealth Scientific Industrial Research Organisation; RDA, recommended dietary allowance.
Folic Acid Deficiency-induced Chromosome Damage and MTHFR C677T polymorphism.

were to (a) validate Buds and NPBs within the CBMN assay as biomarkers for folic acid deficiency and genomic instability; (b) to use this comprehensive set chromosome damage and cell death biomarkers in the CBMN assay to determine the optimal folic acid concentration for in vitro genomic stability; and (c) to test the hypothesis that the MTHFR C677T polymorphism is protective against genomic instability induced by folic acid deficiency in vitro.

Materials and Methods

Approval for this study was obtained from CSIRO Health Sciences and Nutrition and Adelaide University human ethics committees.

Ten volunteers homozygous for the MTHFR C677T polymorphism (TTs) and 10 volunteers age-, gender-, and genotype-matched controls without the polymorphism (CCs) participated. This polymorphism was detected using the method of Frosst et al. (15). Presence of the MTHFR A1298C and methionine synthase A2756G polymorphisms was also previously determined using the methods of van der Put et al. (18) and Leclerc et al. (19), respectively. Volunteers were selected from a database of approximately 200 people who were previously genotyped for these three polymorphisms. CCs and TTs were matched as well as possible for the two other polymorphisms in order to minimize their potential confounding effect. Volunteer characteristics are listed in Table 1.

Volunteers donated approximately 90 ml of blood (lithium-heparin) after an overnight fast on one occasion. All of the blood samples for this study were collected within a 10-week period. Lymphocytes were isolated using Ficol-Paque gradients (Pharmacia Biotech, Uppsala, Sweden) and cultured (5 x 10^5 cells/ml) in 10 ml of medium. Cells were cultured in duplicate in eight 25-ml culture flasks with vented lids (Sarstedt, Adelaide, Australia). Four types of RPMI-1640 (prepared in house) containing 5% dialyzed FCS (Trace Biosciences, Victoria, Australia), 10 units/ml interleukin-2 (Roche Diagnostics, Basel, Switzerland), and 120, 60, 24, or 12 nm folic acid were used.

The cell culture protocol is illustrated in Fig. 2 and has been described in detail elsewhere (4). Briefly, cells were placed in culture, stimulated to divide with PHA (22 μg/ml; Murex Biotech, Kent, England) and then incubated at 37°C and 5% CO₂ in a humidified atmosphere for 9 days. Culture medium was changed 3 and 6 days post-PHA. After 8 days, a 750-μl aliquot of each culture was removed for the cytokinesis block micronucleus assay to give a measure of chromosome damage. Cytochalasin B (4.5 μg/ml; Sigma, St Louis, MO) was added to each tube, and cells were harvested onto microscope slides using a cyto-centrifuge (Shandon Southern Products, Cheshire, United Kingdom) ~30 h later. Slides were then air-dried, fixed, and stained using Diff-Quik (similar results to Wright-Giemsa stain; LabAids, New South Wales, Australia). Slides were prepared from each duplicate culture. Nine days after PHA, cells from the flasks were collected and stored at -80°C and subsequently analyzed for DNA-uracil content as described previously (4). Uracil analyses were performed by J. W. C. and S. M. T. in the Ames laboratory.

Coded slides were scored by one person (J. W. C.) with scores being obtained for each duplicate culture. All viable, apoptotic, and necrotic cells were scored until 500 viable cells were counted. MONO cells were scored for the presence of MNi. Apoptotic and necrotic cells were scored using the criteria proposed by Fenech et al. (20). Briefly, cells with an intact cell membrane and exhibiting chromatin condensation and/or nuclear fragmentation were classified as apoptotic. Cells exhibiting extensive vacuolization and/or a loss of well-defined cytoplasmic and nuclear boundaries were classified as necrotic. NDI was determined as described previously (21). All of the cell culture work, micronucleus assays, and data analysis were performed by J. W. C. in the Fenech laboratory.

A total of 1000 BNed cells were scored for the presence of MNi (Fig. 3A) and NPBs (Fig. 3B) using the criteria described by Fenech (21). BNed cells were also scored for the presence of “Buds,” which may be formed when chromosomes, chromosome fragments, or amplified genes are extruded from the
nucleus. Buds are morphologically similar to MNi; however, they are joined to the nucleus by a nucleoplasmic connection that is clearly thinner than the bud’s diameter (Fig. 3C). Although buds are joined to the main nucleus, buds were scored using the basic size criteria for MNi described by Fenech (21). Briefly, buds must have a diameter not larger than one-third or smaller than one-sixteenth of the diameter of the main nuclei and are usually stained with the same intensity as the main nuclei.

MN, NPB, apoptosis and necrosis data exhibited a normal (Guassian) distribution and the response of these endpoints to folic acid concentration was analyzed using repeated measures one-way ANOVAAs followed by Tukey’s post hoc tests. Nuclear bud data were not normally distributed and were, therefore, analyzed using a Friedman nonparametric test with a Dunn’s posttest. Two-way ANOVA was used to analyze the effect of folic acid concentration and another variable (i.e., genotype or gender) on cellular biomarkers. Two-tailed Pearson correlations were used to analyze relationships between two variables. All of the data are expressed as mean ± SE. The above statistical calculations were performed using GraphPad Prism, version 2.01 (GraphPad Inc., San Diego, CA). A Pearson correlation matrix was performed using Statsoft Stastistica 99, version 5.5 (Statsoft, Tulsa, OK). Significance was accepted at P < 0.05.

Results

Data for the experiment are presented in Table 2 and a correlation matrix is presented in Table 3. When data from CCs and TTs were combined, there was a significant negative correlation between folic acid concentration in the culture medium and all of the markers of chromosome damage. The r² value for MNed BNs, buds, and NPBs in BN cells and MNed MONO cells was 0.55, 0.46, 0.39, and 0.25, respectively (P < 0.0001). These data indicate that DNA damage was minimized at a folic acid concentration of 120 nm.

The percentage of cells that were necrotic correlated negatively with folic acid concentration (P = 0.0023; r², 0.11). Apoptosis did not correlate with folic acid concentration (P = 0.29); however, one-way ANOVA revealed that significantly more apoptosis occurred at 60 nm compared with 120 nm folic acid. NDI was positively correlated with folic acid concentration (P < 0.0001; r², 0.38) and was maximized at a concentration of 60 nm (NDI at 60 versus 120 nm not different; P > 0.05).

The DNA uracil content of cells after 9 days culture was significantly and negatively correlated with folic acid concentration (P < 0.0001; r², 0.19; Ref. 4). Furthermore, DNA uracil content was significantly and positively correlated with the number of MNed BNs (P = 0.0002; r² = 0.17; Fig. 4), BNs with NPBs (P = 0.003; r², 0.12) and BNs with buds (P = 0.0002; r², 0.17). MNed BNs, MNed MONOs, NPBs, buds, and necrosis were all significantly and positively correlated with each other, however the strongest correlations were between MNed BNs, NPBs, and buds (P < 0.001; r², 0.56–0.59).

Two-way ANOVA shows that homozygosity for the MTHFR C677T polymorphism does not affect MNed BNs (P = 0.24), MNed MONOs (P = 0.63), buds (P = 0.22), or NPBs (P = 0.56; Fig. 5). The P for the effect of folic acid concentration in each of these parameters was <0.0001 (Fig. 5). Similarly, apoptosis, necrosis, and NDI were not affected by MTHFR genotype.

Discussion

The data from this experiment clearly show that all of the markers of chromosome damage tested were minimized at a folic acid concentration of between 60 and 120 nm. There was a 2–5-fold higher frequency of these markers in cells grown in medium with 24 nm folic acid relative to 120 nm. Because plasma folate concentrations generally range from approximately 21 to 36 nm in healthy nonanemic individuals (22, 23), these results support the view that there may be some benefit, with respect to genetic stability, in raising blood folate levels. Indeed, studies have shown that relatively short-term folate supplementation can significantly lower baseline levels of genetic damage in humans in vivo (3, 24).

The results of this experiment further emphasize the link between DNA-uracil content and chromosome damage because DNA uracil-content correlates positively with MNi, NPBs, and buds, markers of chromosome breakage, and rearrangement and gene amplification. However, the correlation factor between uracil and MNed BNs suggests that only 17% of the variance in these chromosome damage markers can be ex-

![Fig. 3. Photographs of cells scored in the CBMN assay.](Image)
plained by excessive uracil in DNA. This suggests that other mechanisms such as reduction of DNA methylation or fragile site expression may have also contributed to the induction of mechanisms such as DSBs (3), observed in apoptotic nuclei (28). In this process, Rad 51-recombination protein complexes are formed by a budding process after exposure to γ-irradiation (28). In this study, Buds correlate well with folic acid concentration in the culture medium \( (r^2 = 0.46; P < 0.0001) \). It has now become evident that amplified genes may be eliminated via nuclear budding and MN formation. Shimizu et al. (25, 26) showed that amplified DNA is selectively localized to specific sites at the periphery of the nucleus and eliminated via nuclear budding to form MNs during S phase of mitosis. Furthermore, DNA synthesis inhibitors such as hydroxyurea were shown to increase the rate of elimination of amplified DNA via this process. It was suggested that amplified DNA is eliminated through recombination between homologous regions within amplified sequences forming minicircles of acentric and atelomeric DNA (double minutes) that localize to distinct regions within the nucleus or through the excision of amplified sequences after segregation to distinct regions of the nucleus. The above suggests that the nucleus has a capacity to sense excess DNA that does not fit well within the nuclear matrix, indicating a higher order DNA repair or nuclear housekeeping process. Furthermore, Miele et al. (27) showed that amplified dihydrofolate reductase genes in methotrexate-resistant V79 cells were accumulated in Buds, NPBs, and MNs. It is also reported that MNs are formed by a budding process after exposure to γ-irradiation (28). In this process, Rad 51-recombination protein complexes are detectable throughout the entire nucleus 3 h after irradiation and then concentrate into distinct foci before being extruded from the nucleus into MNs. By using end-labeling, these MNs were shown to exhibit DNA fragmentation analogous to that observed in apoptotic nuclei (28).

Table 2: Experimental results

<table>
<thead>
<tr>
<th>End point</th>
<th>Group</th>
<th>[folic acid] in medium (nM)</th>
<th>12</th>
<th>24</th>
<th>60</th>
<th>120</th>
<th>ANOVA P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>TT</td>
<td>All</td>
<td>CC</td>
<td>TT</td>
</tr>
<tr>
<td>MNed BNs</td>
<td></td>
<td></td>
<td>30.9 ± 2.7</td>
<td>27.2 ± 2.4</td>
<td>29.0 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.98 ± 0.5</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>MNed MONOS</td>
<td></td>
<td></td>
<td>33.6 ± 2.5</td>
<td>21.1 ± 3.2</td>
<td>22.2 ± 2.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.1 ± 0.6</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>BNs + Buds</td>
<td></td>
<td></td>
<td>11.7 ± 1.2</td>
<td>10.5 ± 1.6</td>
<td>11.1 ± 1.0</td>
<td>1.4 ± 0.3</td>
<td>3.5 ± 0.4</td>
</tr>
</tbody>
</table>

- **Apop.** percentage of cells that were apoptotic; **Necr.** percentage of cells that were necrotic; Gp, comparison between groups (CC versus TT). [F], comparison across folic acid concentrations; All, combined data from Ccs and Tts \( (n = 20) \) that were compared across folic acid concentrations using one-way ANOVA. Nonparametric analyses were used for Bud data.

- <sup>a</sup> \( P < 0.05 \) versus 120 nM.
- <sup>b</sup> \( P < 0.05 \) versus 60 nM.
- <sup>c</sup> \( P < 0.05 \) versus 24 nM.
bridges. During cytokinesis these dicentric chromosomes, which span both daughter nuclei, are thought to break unevenly and may form a chromosome with two copies of one or more genes and a chromosome (fragment) with no copies of these genes. The chromatids with multiple copy number of these genes may fuse again during interphase forming a dicentric chromosome (doubling again the gene copy number within the chromosome), which is then replicated during the next nuclear division leading to the next bridge-breakage-fusion cycle and further gene amplification.

The BFB cycle model has been validated as a mechanism for gene amplification in various systems including (a) coformycin-induced amplification of the adenylate deaminase gene in Chinese hamster cells (35); (b) N-(phosphonyacetyl)-L-aspartate-induced amplification of the CAD gene in human fibrosarcoma cells, which is enhanced by the expression of Vpr, a HIV accessory gene (36); (c) methotrexate-induced amplification of the dihydrofolate reductase gene in Chinese hamster cells (37); and (d) actinomycin D-induced amplification of the multidrug resistance 1 gene in Chinese hamster cells through the induction of fragile sites that determined the initiation and size of amplicons (38). Folic acid deficiency-induced fragile site expression and DNA hypomethylation may have also contributed to the promotion of gene amplification and resulted in
elimination of this DNA by nuclear budding in our system. The induction of hypomethylation by 5-aza-2\'-deoxycytidine has been reported to enhance \( \text{N}(\text{phosphonylacetyl})-\text{L-aspartate-} \) induced amplification of the CAD gene in Syrian hamster kidney cells (39).

The scoring of NPBs and Buds is valuable because they have the potential of adding considerably more information to the CBMN assay. For example, the presence of NPB provides a measure of chromosome rearrangement and implies the induction of chromosome breaks that cannot be directly assumed when scoring MNi only, because MNi may arise from both chromosome breaks and chromosome loss. In addition, this study verifies the importance of Buds as a biomarker of DNA damage/gene amplification because of the strong positive correlation with NPBs and MNi and the negative correlation with folic acid concentration. These correlations fit well with the concept that folic acid deficiency may cause gene amplification by initiating BFB cycles in dividing cells. A summary of the events surrounding gene amplification that may be occurring in the micronucleus assay system used in our experiments is presented in Fig. 6.

As described earlier, measures of cytotoxicity are now incorporated into the CBMN assay to give a more thorough indication of the cellular response to the treatment being tested, whether it be nutrient deficiency or exposure to a known mutagen. In this system, apoptosis did not appear to be related to folic acid concentration. Although apoptosis was higher at 60 nM than at 120 nM, the magnitude of this difference is rather small, i.e., 4 cells in 1000, and it is unclear whether this is a physiologically relevant difference. Necrosis was not minimized at 60 nM but at 120 nM, with a small but significant 3% difference. NDI exhibited a strong positive correlation with folic acid concentration but reached a plateau at 60 nM (60 versus 120 nM; \( P > 0.05 \)). Taken together, these results suggest that a folic acid concentration of between 60 and 120 nM is required to minimize cytotoxicity and maximize cell division.

As shown in Table 2, two-way ANOVA analysis indicated that there is no detectable difference in any of the endpoints measured between CCs and TTs. This is also the case for DNA-uracil content (4). These results suggest that lowered uracil incorporation and DNA strand breakage may not significantly contribute to the reduced cancer risk in TTs compared with CCs. The study compared CCs and TTs over a broad physiological folic acid concentration range to maximize the chance of detecting differences between the two groups; however, it is possible that the actual MTHFR activity of the two groups may not have been sufficiently different in this system. As discussed previously (4), there is evidence that high blood riboflavin (vitamin B\(_2\)) concentrations may improve MTHFR activity in TTs (40) because the cofactor for MTHFR, FAD, is a metabolite of riboflavin. Because the C677T polymorphism decreases the enzyme’s binding affinity for FAD and increases the rate of dissociation of FAD from the enzyme (41), it is possible that high concentrations of FAD may improve the MTHFR activity of people homozygous for the polymorphism. The riboflavin concentration of RPMI 1640 is 530 nM, which is

\[ \text{Fig. 6. Summary of genotoxic events that may be induced by folic acid depletion in this in vitro system. * indicates endpoints measured in the comprehensive micronucleus assay presented in this paper.} \]
much higher than the 12–13 nt normally found in plasma (40) and it is, therefore, plausible that this concentration of riboflavin may have increased FAD concentrations enough to restore MTHFR activity. Future research should clarify that the MTHFR activity of cells from CCs and TTs is modified by riboflavin/FAD concentrations in culture medium and in vivo.

If the lack of difference between CCs and TTs seen in this experiment can be explained by the fact that high riboflavin concentration improves MTHFR activity and abolishes the protection afforded by the C677T polymorphism, this begs the question of whether low riboflavin and FAD concentrations may be beneficial in TTs because of a preservation of the polymorphism’s protective effect. It may be interesting to explore the concept of whether current riboflavin RDAs for TTs may need to be carefully evaluated to preserve the protective effect of the C677T polymorphism against some cancers. Similarly, a lower riboflavin intake in CCs may reduce MTHFR activity to a level that produces a net benefit with respect to uracil incorporation into DNA.

In conclusion we have shown that markers of genetic damage, including nuclear budding and NPBs, correlate strongly with the folic acid content of culture medium over a broad physiological concentration range. The link between uracil incorporation and micronucleus formation is also confirmed. The results of this experiment suggest that MTHFR C677T genotype does not influence levels of chromosome damage as measured by the cytokinesis block micronucleus assay. This result does not support the hypothesis that TTs are protected against some cancers because of a diversion of 5,10-MTHF toward thymidine synthesis. However, it is becoming clearer that further research is needed to elucidate under what nutrient conditions MTHFR activity is lowered in TTs and whether MTHFR activity is improved and the protective effect of the C677T polymorphism is undone by high blood (or medium) riboflavin concentrations. Because of the importance of DNA damage as a cause of cancer and other diseases of old age, we have suggested a new paradigm for determining RDAs for micronutrients based on optimizing genomic stability (42). The comprehensive in vitro micronucleus assay demonstrated in this article provides an efficient way of investigating optimal micronutrient concentration required to prevent DNA damage in human cells of different genotype.

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References


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