Methylenetetrahydrofolate reductase polymorphism affects the change in homocysteine and folate concentrations resulting from low dose folic acid supplementation in women with unexplained recurrent miscarriages

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ABSTRACT To determine the effects of daily supplementation of 0.5 mg folic acid on homocysteine and folate concentrations, we investigated 49 women with a history of unexplained recurrent miscarriages. A methionine loading test (including the vitamin concentrations of concern) was used preceding and after 2 mo of folic acid intake. Subsequently, these effects were studied after stratification for C677T 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphism. Folic acid supplementation (for 2 mo) reduced the median fasting and delta (after-load minus fasting) total plasma homocysteine (tHcy) concentrations 27% ($P < 0.001$) and 14% ($P < 0.05$), respectively. Median serum and red cell folate concentrations increased 275 and 70%, respectively ($P < 0.01$). The homocysteine-lowering effect was most marked

in women with the highest tHcy concentrations at baseline. All MTHFR-genotypes (homozygous T/T, n = 8; heterozygous T/C, n = 23; wild type C/C, n = 18) had a different response to the supplementation. After 2 mo, homozygous women showed the greatest decline in median fasting (-41 %; $P < 0.01$) tHcy concentrations, but the lowest absolute increase in serum folate concentration (+26 nmol/ L; $P < 0.05$). In conclusion, 2 mo of daily supplementation of 0.5 mg folic acid in women with a history of unexplained recurrent miscarriages caused, in general, substantially reduced tHcy concentrations. This effect was most distinct in women with the highest tHcy concentrations at baseline and in women homozygous for the 677 C-T mutation of the MTHFR-gene. J. Nutr. 128: 1336-1341, 1998.

KEY WORDS: homocysteine 5,10-methylenetetrahydrofolate reductase folic acid recurrent miscarriages humans

Disturbed methionine-homocysteine metabolism resulting in mild hyperhomocysteinemia has been reported as a risk factor for occlusive arterial and thrombotic diseases (Boushey et al. 1995, Den Heijer 1995), neural tube defects (NTD)4 (Steegers-Theunissen et al. 1994), placental pathology (Goddijn Wessel et al. 1996), pre-eclampsia (Dekker et al. 1995) and unexplained recurrent miscarriages (Wouters et al. 1993). Therefore, lowering total plasma homocysteine (tHcy) concentrations may reduce the risk for these cardiovascular and obstetrical events.

Homocysteine is a demethylated derivative of the essential amino acid methionine. Three vitamins are involved in the homocysteine metabolism: pyridoxal 5'-phosphate (PLP), an active form of vitamin B-6 (transsulfuration pathway), folic acid and cobalamin (vitamin B-12) in the remethylation of homocysteine to methionine. Previous reports showed that supplementation of these B-vitamins is effective in lowering tHcy concentrations before and after methionine load. The effects of combined B-vitamin therapy with folic acid doses varying from 0.4 to 10 mg (Brattstrom et al. 1990, Glueck et al. 1995, Ubbink et al. 1993a, 1993b, 1994 and 1995, Van den Berg et al. 1995) were reported extensively. Furthermore, the effect of folic acid therapy alone was reported for

several populations. In these studies, different doses (0.2-10 mg) and treatment periods (2 wk to 7 mo) were investigated (Guttormsen et al. 1996, Kang et al. 1991, Landgren et al. 1995, Ubbink et al. 1994).

In addition to being influenced by vitamin concentrations, tHcy concentrations are a variable of the common 677 C-T mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene (Jacques et al. 1996). MTHFR is the enzyme responsible for converting 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate (5-MTHF). 5MTHF in turn is the primary methyl donor in the conversion of homocysteine to methionine and is also the primary circulatory form of folic acid. The homozygous (T/T) genotype is associated with thermolability of MTHFR, which leads to a different distribution of the various folate derivatives (Guttormsen et al. 1996, Harmon et al. 1996, Nelen et al. 1997b, Van der Put et al. 1995) and to higher tHcy concentrations (Guttormsen et al. 1996, Harmon et al. 1996, Jacques et al. 1996, Nelen et al. 1997b, Van der Put et al. 1995). Moreover, homozygosity was recently reported to be a risk factor for recurrent miscarriages (Nelen et al. 1997a) and for other diseases associated with hyperhomocysteinemia (Kluijtmans et al. 1997, Sohda et al. 1997, Van der Put et al. 1997). Because of the stronger inverse correlation between tHcy and folate concentrations in T/T women ($r = -0.8$) compared with the two other genotypes ($r = -0.4$) (Nelen et al. 1997b), it can be expected that the T/T genotype is linked to the strongest reduction after folic acid supplementation. Recently, Malinow et al. (1997) reported a larger decrease in fasting tHcy concentrations in T/T individuals (coronary heart disease patients and their controls) than in the other two genotypes after 3 wk of 1 or 2 mg folic acid supplementation.

Several reports, in particular that of Czeizel and Dudas (1992), have demonstrated that periconceptional vitamin use decreases the first occurrence of NTD. Consequently, in many countries, including the Netherlands, women intending to become pregnant are being advised to use 0.5 mg folic acid daily =>4 wk before conception, continuing until wk 9 of pregnancy. Apparently, before studying the effect of supplementation on the incidence of recurrent miscarriages, the effects of this specific folic acid dosage on

folate and homocysteine metabolism in these women should be established. Furthermore, the effect of folic acid supplementation as a variable of the MTHFR-genotype in these women is of interest. We thus investigated the effects of daily 0.5 mg folic acid supplementation on tHcy and folate concentrations in women with a history of recurrent miscarriages before and after stratification for the C677T MTHFR polymorphism.

SUBJECTS AND METHODS

Subjects. We investigated 49 Caucasian women suffering from unexplained recurrent miscarriages, who had been referred to our hospital between September 1994 and September 1996 and who were included in a study on determination of the prevalence of the 677C-T mutation (Nelen et al. 1997a). Spontaneous miscarriage was defined as early pregnancy loss within 16 wk of menstrual age, thereby excluding ectopic pregnancy and elective abortion. Recurrent miscarriages were defined as at least two consecutive spontaneous miscarriages after conceiving from the same partner. Thirty-seven women (76%) experienced three or more consecutive miscarriages. All early pregnancy losses (n = 160) were confirmed either histologically (52%) or by a positive routine urinary hCG test (>50 lu/L) and/or by ultrasound imaging.

In all of the women studied, chromosomal rearrangements, severe uterine anomalies, thyroid dysfunction, glucose intolerance and immunologic disorders were excluded by routine investigational procedures as previously described (Wouters et al. 1993). Furthermore, women who had been diagnosed with severe vitamin B-12 deficiency (serum concentration <100 pmol/L) or either renal or liver disease were excluded. All of the women included were in good general health and gave written informed consent before participation. The study was approved by the Institutional Review Board of the University Hospital Nijmegen.

Methods. Methionine-homocysteine metabolism was investigated by a standardized oral methionine-loading test before and after 2 mo (range 44-79 d) of folic acid supplementation. After an overnight fast, venous blood samples were drawn to measure

tHcy and vitamin concentrations. Then L-methionine, 0.1 g/kg body weight, was administrated orally in 200 mL of orange juice. All women ate a standardized methionine-restricted breakfast and luncheon. Only coffee, water and tea without milk were allowed as drinks during the test period. Six hours after the methionine loading, venous blood was collected for measuring the (after-load) tHcy concentration. At the time of measurement, none of the women was pregnant, lactating, taking oral contraceptives or was on medication possibly interfering with methionine-homocysteine metabolism. None of the women had used B-vitamin supplements for at least 6 mo before the first measurement.

Folic acid administration (0.5 mg daily) started the day after the first methionine-loading test. Treatment compliance was verified by history and diary cards. After 4 mo of supplemrntation (range 96140 d), the measurements were repeated in 20 women (41%). The other 29 women were not investigated further because they had conceived (n = II), did not return for follow-up visit due to social or personal reasons (n = 14) or their treatment period was <4 mo (n = 4) on September 1, 1996.

Blood samples for measurement of total homocysteine concentrations in plasma were drawn in 4-mL EDTA vacutainer tubes and centrifuged within 30 min at 3000 x g for 10 min. The plasma was separated and immediately stored at -20 degC. Total homocysteine concentrations were measured by HPLC technique and fluorimetric detection (Te PoelePothoff et al. 1995). Dry and heparinized 10-mL vacutainer tubes were used for collecting venous blood samples to assay PLP (whole blood), vitamin B-12 (serum) and folate (serum and red cells) concentrations. Determination of PLP was performed by HPLC technique (Schrijver et al. 1981). The distribution of whole blood PLP is non-Gaussian (calculated skewness: 3.5) with a normal range between 29 and 76 nmol/L (2.5-97.5 percentile) and a median of 53 nmol/L as previously reported (Goddijn Wessel et al. 1996, Wouters et al. 1995). Vitamin B-12 and folate concentrations were measured simultaneously with Dualcount Solid Phase Boil Radioassay (Diagnostic Products, Los Angeles, CA), as previously described (Mooij et al. 1991).

The 677 C-->T mutation was investigated by polymerase chain reaction (PCR) of a genomic DNA fragment followed by restriction enzyme analysis with HinFI (Frosst et al. 1995).

Statistics. Data are presented as medians (range) because the tHcy and folate concentrations demonstrated a skewed distribution. Therefore all statistical comparisons for paired data were performed using Friedman's two-way ANOVA and Wilcoxon's signed-rank test. To compensate for the lack of a placebo group, a P-value <0.01 was considered to be significant. In the case of unpaired data, the MannWhitney U Test was used and $P < 0.05$ was considered to be significant. Statistical analyses were performed with Astute Statistics Add In for Microsoft Excel (1993 DDU Software, The University of Leeds, UK).

RESULTS

Demography. The participating women had a median age of 33.5 y (range 21.8-41.0 y) and a median body mass index of 23.0 kg/mr (range 20.0-36.2 kg/m2). Forty-seven percent (n = 23) of the women with recurrent miscarriages were secondary aborters, meaning they had given birth to at least one liveborn child. The remaining 26 women had suffered solely from recurrent miscarriages (primary aborters). In total, these 49 women had experienced 195 pregnancies with a median of four pregnancies per woman (range 2-7).

Effects of folic acid supplementation on biochemical parameters. THcy and vitamin concentrations at the different sampling time points are given as medians (range) in Table 1. After 2 mo of folic acid supplementation, the median fasting and delta (after-load minus fasting) tHcy concentrations were reduced 27% ($P < 0.01$) and 14% ($P = 0.048$), respectively. Median serum (+275%) and red cell (+70%) folate concentrations increased significantly. Between 2 and 4 mo of supplementation, the red cell folate concentration increased an additional 36% to a median of 1200 (range 750-1500 nmol/L) ($P < 0.01$). At the same time, no further significant change in tHcy concentration

was observed. During the period of folic acid supplementation, no significant changes were observed in the median concentrations of PLP or vitamin B-12.

The fasting and delta tHcy concentrations measured before folic acid supplementation were divided into tertiles as follows: fasting: high >14.5 (mu)mol/L, medium 12.1-14.5 /mol/L and low < 12.1 (mu)mol/L; for delta tHcy concentrations: high >28.3 (mu)mol/L, medium 20.1-28.3 /,mol/L and low <20.1 (mu)mol/L. The effects of folic acid supplementation on tHcy concentrations in the different groups are described in Table 2. After 2 mo of low dose folic acid supplementation, 94% of the women in the fasting high-group had a tHcy concentrations <14.5 (mu)mol/L. None of the women in the fasting medium or low group shifted to the high group during the supplementation period. Fifty-three percent of the women in the delta high group still had a change in tHcy concentration of >28.3 (mu)mol/ L after 2 mo of supplementation; one woman in the delta low group and three women in the delta medium group shifted to the high group.

Effects of folic acid supplementation for each C677T MTHFR-genotype. The women were categorized according to their genotype of the 677 C-T mutation in the MTHFR gene as follows: homozygous genotype (T/T), n = 8; heterozygous genotype (T/C), n = 23; and the wild type (C/C), n = 18. The three groups were comparable in median age and number of miscarriages. The respective tHcy and B-vitamin concentrations are shown in Table 3. Before folic acid supple mentation, we found a significant difference in fasting tHcy concentration between the T/T and the T/C genotype ($P < 0.05$). Furthermore, the serum folate concentration was significantly lower in homozygous women compared with the T/ C ($P < 0.01$) and C/C genotypes ($P < 0.01$). No significant differences were observed between the T/C and C/C genotypes. The effects of folic acid supplementation for each genotype are shown in Table 3.

After 2 mo of folic acid supplementation, homozygous women continued to have significantly lower median serum folate concentrations (35 nmol/L) compared with the T/C (45 nmol/L) and C/C genotypes (51 nmol/L) (Fig. 1). Furthermore, they had the

lowest fasting tHcy concentration, but differences were not significant (P = 0.10; Fig. 1). For all other variables studied, the three genotypes did not differ. After 4 mo of folic acid supplementation, there were no significant differences in serum folate concentrations among the three genotype groups.

The effects of folic acid supplementation on tHcy and folate concentrations are presented as median changes (concentration after 2 mo minus the concentration before supplementation)(Table 3). Homozygous women showed the strongest decline in fasting tHcy concentrations as a result of folic acid supplementation. The lowering effect in homozygous women was -6.85 (-3.3 to -10.9) (mu)mol/L vs. -2.4 (-0.5 to -7.4) (mu)mol/L in heterozygous women and -3.3 (-0.7 to -13.0) (mu)mol/L in wild type women. This change was significantly stronger in T/T individuals than in the other two genotypes ($P < 0.05$). For delta tHcy concentrations, there was no significant difference in decline between the three genotypes ($P > 0.05$). The increase in serum folate concentrations was significantly different between the T/T and C/C genotypes ($P < 0.05$). However, there was no significant difference among the three genotypes in median increase of red cell folate concentration. Between the T/C and C/C individuals, no significantly different changes were found in any variable studied in response to folic acid supplementation.

Discussion

Daily folic acid supplementation for 2 mo reduced tHcy concentrations substantially (Table 1). Women with the highest tHcy concentrations before folic acid supplementation, who have possibly the highest risk for cardiovascular disease, NTD or a subsequent miscarriage, showed the strongest decrease. This is in accordance with the effects reported by others (Guttormsen et al. 1996, Landgren et al. 1995, Mason and Miller 1992, Ubbink et al. 1994).

Almost all women (94%) in the fasting high group had tHcy concentrations in the medium and low range after 2 mo of low dose folic acid supplementation (Table 2). This effect may indicate that high fasting tHcy concentration in these women is not caused

by a blockade of the homocysteine transsulfuration pathway, but is due to a defective remethylation of homocysteine into methionine. Impaired remethylation can be caused by either decreased methionine synthase activity as a result of vitamin B-12 deficiency or reduced serine hydroxymethyltransferase activity due to vitamin B-6 inadequacy or less 5-MTHF production by lower MTHFR activity. Apparently, the first two of these remethylation pathways did not contribute to the effect of folic acid supplementation as demonstrated here. In regard to the first pathway, women with severe vitamin B-12 deficiency were excluded from this study, and no significant changes in median vitamin B-12 concentrations were observed. In regard to vitamin B-6 inadequacy, only one of the 17 fasting high group women started the study with a whole blood PLP concentration <29 nmol/L, which normalized during the supplementation period; in contrast, in another woman, PLP concentration fell to a value below this normal range. Our observations emphasize the importance of the 677 C-T mutation in the MTHFR-gene. Women homozygous for this mutation showed the highest fasting tHcy concentrations and the lowest serum folate concentrations before supplementation compared with the T/C and C/C genotypes, which is in line with previous reports (Guttormsen et al. 1996, Harmon et al. 1996, Jacques et al. 1996, Nelen et al. 1997b, Van der Put et al. 1995). After 2 mo of low dose folic acid supplementation, fasting tHcy concentrations of the T/T genotype women equaled those of the other genotypes. Because the product of MTHFR, 5-MTHF, is the primary circulatory form of folic acid, the phenotype of reduced enzyme activity is expressed as lower folate concentrations even after shortterm folic acid supplementation in homozygous women.

Homozygous women lowered their fasting tHcy concentrations by >40% when they received low dose folic acid supplementation of 0.5 mg/d for 2 mo (Table 3). Recently, Jacques et al. (1996) reported that the homozygous genotype for the 677C-T mutation in the MTHFR-gene results in elevated tHcy concentrations if folate concentrations are at the low end of the normal range, whereas homozygous individuals with normal or high folate concentrations usually have normal tHcy concentrations. In light of this observation, it is of interest whether tHcy can be lowered by diet changes alone in women with hyperhomocysteinemia, based on homozygosity for the 677C-T mutation in

the MTHFR-gene. Because of the demonstrated slower response pattern to folic acid supplementation in T/T women, such an effect could be evaluated only after a sufficiently long study period.

The effect of folic acid supplementation on delta tHcy concentrations is less striking. After 2 mo of supplementation, only 47% of women in the delta high group reduced their tHcy concentrations to concentrations comparable to the medium or low range. The persistent high delta tHcy concentrations in 53% of the women during supplementation suggest a blockade of the homocysteine transsulfuration pathway and thus may indicate the need of additional vitamin B-6 supplementation.

A limitation of our study is the lack of a placebo group. Because Dutch authorities recommend folic acid supplementation to all women intending to become pregnant, which was the case in all participants, a placebo-controlled study design was ethically not feasible. The MTHFR-genotype-related effects of low dose folic acid supplementation discovered in this study population are comparable to those found by others (Malinow et al. 1997); they are theoretically plausible and probably not affected by the study design used. The 41% lowering of the fasting tHcy concentration in T/T women is higher than the previously reported 21% (Malinow et al. 1997). This may be due to differences in the supplementation period (2 mo vs. 3 wk) and in the starting fasting tHcy concentrations of the T/T individuals (a geometric mean of 13.3 (mu)mol/L vs. 11.8 (mu)mol/L).

We also studied the effect of low dose folic acid supplementation during 4 mo in a subset of our study group. After 2-4 mo of folic acid use, an additional increase of serum and red cell folate concentrations with no further decrease of tHcy was noted. Because the mean life span of a red cell is - 120 d, this effect on the red cell folate concentrations can be expected because folate concentration in erythrocytes reflects the folate status during erythropoiesis (Heseker and Schmitt 1987). In conclusion, 2 mo of daily supplementation of 0.5 mg folic acid reduced median fasting and delta tHcy concentrations 27 and 14%, respectively. This low dose folic acid supplementation

provided the strongest decline of tHcy concentrations in women homozygous for the 677 C-T mutation in the MTHFR-gene.

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2 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact. 3 To whom correspondence and reprint requests should be addressed. 4 Abbreviations used: MTHFR, 5,10-methylenetetrahydrofolate reductase; 5MTHF, 5-methyltetrahydrofolate; NTD, neural tube defects; PCR, polymerase chain reaction; PLP, pyridoxal 5'-phosphate; tHcy, total plasma homocysteine.

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