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Research paper

Formate supplementation enhances folate-dependent nucleotide biosynthesis and prevents spina bifida in a mouse model of folic acid-resistant neural tube defects

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ABSTRACT

The *curly tail* mouse provides a model for neural tube defects (spina bifida and exencephaly) that are resistant to prevention by folic acid. The major *ct* gene, responsible for spina bifida, corresponds to a hypomorphic allele of *grainyhead-like 3* (*Grhl3*) but the frequency of NTDs is strongly influenced by modifiers in the genetic background. Moreover, exencephaly in the *curly tail* strain is not prevented by reinstatement of *Grhl3* expression. In the current study we found that expression of Mthfd1L, encoding a key component of mitochondrial folate one-carbon metabolism (FOCM), is significantly reduced in *ct/ct* embryos compared to a partially congenic wild-type strain. This expression change is not attributable to regulation by *Grhl3* or the genetic background at the *Mthfd1L* locus. Mitochondrial FOCM provides one-carbon units as formate for FOCM reactions in the cytosol. We found that maternal supplementation with formate prevented NTDs in *curly tail* embryos and also resulted in increased litter size. Analysis of the folate profile of neurulation-stage embryos showed that formate supplementation resulted in an increased proportion of formyl-THF and THF but a reduction in proportion of 5-methyl THF. In contrast, THF decreased and 5-methyl THF was relatively more abundant in the liver of supplemented dams than in controls. In embryos cultured through the period of spinal neurulation, incorporation of labelled thymidine and adenine into genomic DNA was suppressed by supplemental formate, suggesting that de novo folate-dependent biosynthesis of nucleotides (thymidylate and purines) was enhanced. We hypothesise that reduced Mthfd1L expression may contribute to susceptibility to NTDs in the *curly tail* strain and that formate acts as a one-carbon donor to prevent NTDs.

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1. Introduction

The network of reactions that comprises folate one-carbon metabolism (FOCM) supplies one carbon units for a number of downstream biosynthetic pathways including nucleotide biosynthesis and methylation reactions [1,2]. Corresponding with the crucial role of FOCM in several cellular functions, abnormal FOCM is associated with a range of diseases, including cancers, fatty liver disease, inborn errors of metabolism, autism, age-related cognitive impairment and birth defects, particularly neural tube defects (NTDs). NTDs, including spina bifida and anencephaly, are a group of birth defects that result from incomplete formation of the neural tube which is the precursor of the brain and spinal cord in the developing embryo [3]. The causes of most NTDs in humans are not well understood owing to their complex etiology which is thought to involve multiple genetic and environmental factors [4]. Sub-optimal maternal folate status is associated with increased risk of an NTD-affected pregnancy while maternal supplementation with folic acid reduces susceptibility [5], although some NTDs are not prevented ('folic acid-resistant'). Polymorphisms and/or variants in some FOCM-related genes (e.g. *MTHFR*, *MTHFD1L*, *AMT* and *GLDC*) have been associated with NTDs [3,6], while abnormal thymidylate

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biosynthesis was identified in a subset of cell lines from NTD patients [7]. Together these data suggest that there is a contribution of abnormal FOCM to NTDs, although it is currently unclear whether this corresponds to NTDs that are preventable by or resistant to folic acid.

Among mouse models of NTDs, some are responsive to folic acid, including *splotch* (*Sp*^{2H}, *Pax3*) and *Cited2* null strains [8–10], while others are resistant [11]. Among the latter group, the *curly tail* (*ct*) strain has been studied extensively as a model for human NTDs [12]. Homozygous *ct/ct* embryos develop partially penetrant NTDs comprising spina bifida and/or exencephaly. These defects arise due to failure in completion of neural tube closure in the spinal and cranial regions respectively. Open NTDs only affect a proportion of mutant embryos but even where neural tube closure is completed around 50% of mice develop tail flexion defects owing to delay in spinal closure. The major *ct* gene corresponds to a hypomorphic allele of *Grhl3*, encoding the grainyhead-like 3 transcription factor [13,14]. However, penetrance of defects is strongly influenced by genetic background [15]. For example, a polymorphic variant of *Lmnb1* was found to influence the frequency of both cranial and spinal NTDs [16].

In addition to genetic modifiers, the frequency of NTDs in the *ct* strain is influenced by several environmental factors, including retinoic acid, inositol and hyperthermia [17–19]. However, there is no protective effect of folic acid [12,20]. Supplemental folic acid does not prevent NTDs in *ct/ct* embryos. However, they are sensitive to maternal dietary folate deficiency, which causes a significant increase in the frequency of cranial NTDs and a delay in overall growth and developmental progression, both in *ct* mice and in a genetically matched wild-type strain ($+^{ct}$) [21]. The same dietary model does not cause NTDs in other wild-type strains [21–23], suggesting the presence of predisposing modifier genes in the *ct* genetic background. Analysis of cultured fibroblasts did not indicate a defect of thymidylate biosynthesis in the *ct* strain, but abnormalities of FOCM were observed [24]. For example, the SAM/SAH ratio is lower in *ct/ct* than in $+^{ct}/+^{ct}$ embryos at E10.5, owing to increased abundance of SAH. Moreover, folate-deficiency led to an increase rather than a decrease in SAM/SAH as observed in wild-type and other strains. Diminished methylation appeared unlikely to contribute to NTDs in *ct/ct* embryos as introduction of an *Mthfr* null allele did not increase the frequency of NTDs; no NTDs were observed among *ct/ct*;*Mthfr*$^{-/-}$ embryos [24].

Although FA does not prevent NTDs in *curly tail* mice, we found that intervening downstream of FOCM, at the level of nucleotide biosynthesis may influence the rate of NTDs. Hence, combinations of thymidine and adenine or GMP resulted in a significant protective effect [25]. In the current study we further investigated the potential contribution of altered FOCM to NTDs in the *ct* strain.

2. Materials and methods

2.1. Mice

Curly tail (*Grhl3*^{ct}) and partially congenic wild-type strains ($+^{ct}/+^{ct}$) were maintained as closed random-bred colonies [12,16]. Wild-type mice for plasma and urine analysis were on a mixed CBA/101 background. The transgenic *curly tail* line (*Grhl3*^{ct}/*Grhl3*^{ct}; Tg(*Grhl3*)1Ndeg/0), here denoted *ct/ct*$^{TgGrhl3/0}$, carries a BAC that encompasses the *Grhl3* gene, as described previously [13]. Mice carrying a conditional (floxed) allele of *Grhl3* have been described [26]. These mice were crossed to β-actin-*Cre* mice to generate heterozygous null, *Grhl3*$^{+/-}$, mice used in experimental matings in this study. *Curly tail* mice are maintained as a homozygous colony. Other mice and embryos were genotyped by PCR of genomic DNA, as described in the relevant original publications [13,26].

Animal studies were carried out under regulations of the Animals (Scientific Procedures) Act 1986 of the UK Government, and in accordance with guidance issued by the Medical Research Council, UK in *Responsibility in the Use of Animals for Medical Research* (July 1993).

2.2. Supplementation and collection of embryos

Experimental litters were generated by overnight mating. On detection of a copulation plug the following morning, dams were separated and litters designated embryonic day (E) 0.5. Treatments were 20 mg/ml or 30 mg/ml sodium formate in drinking water and control (water only). Doses were based on previous studies [27,28]. Formate treatment was started from E0.5 and continued until litters were collected, between E10.5 and 12.5. Embryos were dissected from the uterus in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and assessed for the presence of NTDs under a light microscope. Resorptions were recorded and the crown-rump (CR) length measured using an eyepiece graticule. Embryos were rinsed in PBS and stored at −80 °C.

2.3. Quantitative real time RT-PCR and sequencing

RNA was isolated using TRIzol (ambion) and DNA removed by DNase I digestion (DNA-Free, ambion). First strand cDNA synthesis was performed using random hexamers (Superscript VILO cDNA synthesis kit) and RT-qPCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) on a C1000 Touch Thermal Cycler (Bio-Red). Primers for *Mthfd1l* were: 5′-TCATGGCCGTGCTGGCCTTG-3′ and 5′-TGGCAAAGGGACCAGCGTG-3′ and primers for *Grhl3* were 5′-CCAGACTCCAGTAACAATG-3′ and 5′-AAGGGTGAGCAGGTTCGCTT-3′. Each sample was analysed in triplicate and results were normalised to *Gapdh* mRNA abundance as previously [13,16]. Similar results were obtained when *Beta-actin* was used for normalisation.

The coding sequencing of *Mthfd1L* was sequenced in *ct/ct* and $+^{ct}/+^{ct}$ strains (2 independent embryos for each strain) using cDNA prepared from E10.5 embryos (as above). A series of 8 overlapping primer pairs were used to amplify the entire coding region (28 exons), as well as flanking sequence (100 bp of the 5′ UTR and 210 bp of the 3′ UTR). PCR products were purified using QIAquick PCR Purification Kit (Qiagen). Products were sequenced in forward and reverse directions by Sanger Sequencing (Source Bioscience) and sequences were analysed using Sequencher (GeneCodes Corporation) and compared to the reference Mthfd1L sequence (GenBank XM_006512449.2).

2.4. Microsatellite and SNP genotyping

A series of 18 microsatellite markers (D10Mit84, D10Mit80, D10Mit245, D10Mit123, D10Mit306, D10Mit279, RH125020, RH126580, AI265638, 236300, AI317366, AW536662, PMC25853P1), 236299, AU018232, D10Mit49 and 236303) and 4 SNPs (rs47265432, rs249746523, rs218957174, rs217495350) flanking and within *Mthfd1L* on mouse chromosome 10, were tested for polymorphism between genomic DNA of SWR and *curly tail* strains. Polymorphisms were detected either by size difference between microsatellites or by sequencing of PCR products. A synonymous coding SNP rs47265432 was informative. The sequence at this SNP was determined by sequencing of a 432 bp PCR product generated by primer pair 5′-CTCCAGCACTGAGACCCTCT and 5′-TGCTCCACCCTACCTGACTC.

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