



Inhibition of thymidylate synthase affects neural tube development in mice

Xiuwei Wang^{a,1}, Zhen Guan^{a,1}, Yanting Dong^c, Zhiqiang Zhu^a, Jianhua Wang^{a,*},
Bo Niu^{a,b,*}

^a Beijing Municipal Key Laboratory of Child Development and Nutriomics, Capital Institute of Pediatrics, Beijing 100020, China

^b Department of Biochemistry and Molecular Biology, Shanxi Medical University, Taiyuan 030001, China

^c The Respiratory Department, The Second Hospital of Shanxi Medical University, Taiyuan 030001, China

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ABSTRACT

Thymidylate synthase (TYMS) is a key enzyme in the de novo synthesis of 2'-deoxythymidine-5'-monophosphate (dTMP) from 2'-deoxyuridine-5'-monophosphate (dUMP). Our aim was to investigate the role of dTMP dysmetabolism via inhibition of TYMS by an inhibitor, 5-fluorouracil (5-FU) in the occurrence of neural tube defects (NTDs). We found that a high incidence of NTDs occurred after treatment with 5-FU at 12.5 mg/kg body weight. TYMS activity was significantly inhibited with decreased dTMP and accumulation of dUMP after 5-FU injection. The proliferation of neuroepithelial cells were markedly inhibited in NTDs compared with control. Expressions of proliferating cell nuclear antigen and phosphorylated histone H3 were significantly decreased in NTDs, while phosphorylated replication protein A2, P53 and Caspase3 were significantly increased in NTDs compared with control. These results indicated that inhibition of TYMS affected the balance between proliferation and apoptosis in neuroepithelial cells, which might shed some lights on the mechanisms involved in NTDs.

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1. Introduction

Folate deficiency is closely related to the development of neural tube defects (NTDs) [1–4]. However, the causal metabolic pathway between folate deficiency and NTDs remains unknown. Folic acid, as a carrier of one carbon unit, plays a vital role *in vivo*. It is involved in numerous cellular development processes including the synthesis of purines and thymidylate (dTMP), the formation of the primary methyl donor for the methylation of DNA, histones, proteins and lipids [5,6]. Among these development processes, DNA synthesis is one of the most important pathways related to folate. However, whether dTMP dysmetabolism is one of the major mechanisms in folate deficiency inducing NTD embryos requires further investigations. In our previous study, NTD embryos were induced by inhibition of dihydrofolate reductase (DHFR), a key enzyme in folate metabolic pathway. The results demonstrated that methotrexate (MTX) induced apoptosis and slowed proliferation by interference of folate metabolism [7,1–4]. However, the exact mechanism concerning the relationship of apoptosis and proliferation or their correlated pathways with NTDs induced by folate deficiency needs

to be further clarified. Here, our study investigated the role of dTMP metabolism in the development of neural tube.

The fact that cellular folate deficiency diminished dTMP synthesis has been demonstrated in different models [9,10,11,12]. Decreased dTMP levels caused impaired DNA synthesis and the accumulation of dUMP which may misincorporates into DNA and disturbs DNA synthesis [13,14,15]. In our study, we aim to establish a mouse model of NTDs via disturbing the dTMP metabolism by 5-fluorouracil (5-FU). 5-FU is rapidly metabolized to fluoro-deoxyuridine monophosphate, a specific binding inhibitor to TYMS. Inhibiting of TYMS appears to be the primary antitumor mechanism of 5-FU, which results in depletion of thymidine pools, impairment of DNA synthesis, and reduction of cell proliferation. However, abnormal DNA synthesis in NTDs induced by 5-FU has not been reported. In this study, NTD embryos were induced by inhibition of TYMS following intraperitoneal injection of 5-FU into Gestational Day (GD) 7.5 mice. We investigated the potential mechanisms of NTD occurrence including dTMP metabolism and proliferation using this model.

* Corresponding authors at: Capital Institute of Pediatrics, Beijing 100020, China.

E-mail addresses: fywjh@163.com (J. Wang), niub2004@126.com (B. Niu).

¹ Xiuwei Wang and Zhen Guan contributed equally to this manuscript.

2. Materials and methods

2.1. Animals and 5-FU treatment

C57BL/6 mice (Vital River Laboratories, Beijing, China) (7–8 week, 19–20 g) were mated according to our previous study [7]. Mice were individually housed under controlled temperatures (22 °C, relative humidity 40–60%) with a 12 h light/dark cycle and with free access to pellet food and tap water ad libitum. Pregnant mice were randomly divided into 8 groups with 8 mice in each group. 5-FU (Sigma-Aldrich Corp. St. Louis, MO USA) was dissolved in 0.9% NaCl and seven groups were intraperitoneally injected with different doses of 5-FU (5, 7.5, 10, 11, 12.5, 15 and 30 mg/kg body weight) with 0.2 mL/10 g body weight on GD7.5. A control group was intraperitoneally injected with 0.9% saline on GD7.5. Pregnant mice were sacrificed and embryos were examined under dissecting microscope at GD13.5. All the procedures of the experiment were approved by Ethics Committee of the Capital Institute of Pediatrics (CIP2009012).

2.2. Examination of the embryos and sample collection

Pregnant mice were euthanized by sodium pentobarbital overdose and their embryos were explanted into Hank's balanced salt solution (Life Technologies Inc., Burlington, ON, Canada). Fetuses were measured, weighed, and examined under a dissecting microscope for external malformations on GD 13.5. Growth retardation was defined as whole embryos with a significant decrease based on weight, crown-rump length or morphological characteristics.

Embryonic tissues from control and 5-FU-treatment group (12.5 mg/kg body weight) were collected for evaluation of real-time quantitative polymerase chain reaction (RT-qPCR), western blot analysis, TYMS activity, dTMP and dUMP levels at different time points after 5-FU treatment. For evaluation of TYMS activity, dTMP and dUMP levels at timepoints up to 48 h after 5-FU injection, whole embryos were used for evaluation; at 96 h after injection (GD11.5), isolated embryonic neural tissues from control and NTD embryos were used. For all timepoints, embryonic tissue samples were pooled from one control and one 5-FU treated litter. Normal embryonic neural tissues from one litter were isolated and pooled as one normal sample, while NTD embryos from one litter were picked, and excised neural tissues were isolated and pooled as one NTD sample. Embryonic neural tissues from the most rostral aspect of the forebrain to the caudal aspect of the hindbrain (above the otic vesicle) were microdissected and checked to eliminate the mesoderm or non-neural tissues as precisely as possible under a dissecting microscope.

For RT-qPCR and western blot analysis, neural tissues were isolated as described above and pooled from six control and six 5-FU treated embryos (12.5 mg/kg body weight) at 96 h after injection. Following removal of neural tissues, the remaining embryonic tissue was also pooled and evaluated.

2.3. TYMS activity, dTMP and dUMP levels assay

TYMS activity was determined according to the previous description [16,17]. The levels of dTMP and dUMP were detected by high performance liquid chromatography (HPLC) according to the previous description [17,18]. Each sample was analyzed at least three times and data are presented as mean \pm SD.

2.4. Analysis of proliferation in neuroepithelial cells

Effect of 5-FU on proliferation of neuroepithelial cells was evaluated by the Click-iT EdU Imaging Kits (Invitrogen, Life Technologies Corporation) according to the manufacturer's instruction. To deter-

mine the status of proliferation after TYMS activity, dTMP and dUMP levels recovered, NTDs and control embryos were collected at 96 h after intraperitoneally injection of 5-FU at the optimal dose (12.5 mg/kg body weight). Six equal sized fields were randomly chosen, and the mean number of positive cells was counted. Data are presented as mean \pm SD.

2.5. RT-qPCR

Total RNA from control and NTD embryonic neural tube tissues (GD 11.5) was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The quantity of RNA was determined by Nanodrop 2000 Spectrophotometer at 260/280 nm 500 ng total RNA was used for each reaction. RT-qPCR was performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA) using Power SYBR Green. Primers for the reaction were showed as followed: *Pcna* forward primer: TCAGGTACCTCAGAGCAAACG, reverse primer: AAGTGGAGAGCTTGGCAATG; *Ptch1* forward primer: AATTCTCGACTCACTCGTCCA, reverse primer: CTCCTCATATTTGGGGCCTT; *Caspase3/Casp3* forward primer: GGGGAGCTTGGAAACGCTAAG, reverse primer: CCACTGACTTGCTCCATGT; *P53* forward primer: CCTCAGCATCTTATCCGA GTGG, reverse primer: TGGATGGTGTTACAGTCAGAGC; *Gapdh* Forward primer: GTCATCCATGACAACCTTGG, Reverse primer: GAGCTGACAAAGTGGTCTG.

2.6. Western-blot analysis

Protein levels of proliferating cell nuclear antigen (PCNA), phosphohistone H3 (PH3), phosphorylated replication protein A2 (p-RPA2), P53 and cleaved caspase-3 were determined. Briefly, proteins from embryonic neural tube tissues of control and 5-FU-treatment embryos with NTDs (12.5 mg/kg body weight) were extracted on GD11.5 using CellLytic Cell Lysis Reagent (Sigma) according to the protocol. Protein concentration was determined by Nanodrop 2000 Spectrophotometer. 40 μ g proteins were transferred to polyvinylidene fluoride (PVDF) membranes after the sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and then sealed by 5% skim milk at room temperature for 1 h. First antibodies PCNA antibody (abcam, 1: 500), PH3 (Ser10) antibody (Cell Signaling Technology, 1: 500), p-RPA2 antibody (Cell Signaling Technology, 1:500), P53 antibody (abcam, 1:750) and cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology; 1: 250) were respectively added to the membrane overnight and then secondary antibodies (Cell Signaling Technology; 1:1000) were added for 2 h. ECL was used to visualize the detected proteins and densitometry was measured by use of Gel-Pro analyzer software.

2.7. Statistical analysis

All data are expressed as mean \pm SD. Analyses were performed using SPSS13.0 (SPSS Inc., Chicago, IL, USA) software with the critical α -level set at $P < 0.05$. Statistical analyses were examined by use of Student's *t*-test and Wilcoxon rank-sum test.

3. Result

3.1. Effects of 5-FU on the development of neural tube in mice

In order to investigate the effects of TYMS inhibition on embryonic neural tube development by 5-FU, pregnant mice were intraperitoneally injected with different doses of 5-FU (5, 7.5, 10, 11, 12.5, 15 and 30 mg/kg body weight) on GD7.5, which is a critical period for murine neural tube closure. On GD13.5 embryos were

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