DNA methyltransferase mediates dose-dependent stimulation of neural stem cell proliferation by folate

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Abstract

The proliferative response of neural stem cells (NSCs) to folate may play a critical role in the development, function and repair of the central nervous system. It is important to determine the dose-dependent effects of folate in NSC cultures that are potential sources of transplantable cells for therapies for neurodegenerative diseases. To determine the optimal concentration and mechanism of action of folate for stimulation of NSC proliferation in vitro, NSCs were exposed to folic acid or 5-methyltetrahydrofolate (5-MTHF) (0–200 μmol/L) for 24, 48 or 72 h. Immunocytochemistry and methyl thiazolyl tetrazolium assay showed that the optimal concentration of folic acid for NSC proliferation was 20–40 μmol/L. Stimulation of NSC proliferation by folic acid was associated with DNA methyltransferase (DNMT) activation and was attenuated by the DNMT inhibitor zebularine, which implies that folate dose-dependently stimulates NSC proliferation through a DNMT-dependent mechanism. Based on these new findings and previously published evidence, we have identified a mechanism by which folate stimulates NSC growth.

Keywords: DNA methyltransferase; Neural stem cells; Folic acid; 5-Methyltetrahydrofolate; Proliferation

1. Introduction

Folates are enzyme cofactors in one-carbon metabolism (OCM) that play critical roles in the development, function and repair of the central nervous system (CNS) [1,2]. Strong epidemiological and experimental evidence links disorders of OCM to neurodegenerative and neuropsychiatric diseases, including neural tube defects, Alzheimer’s dementia and depression [3,4]. CNS cells are largely dependent on systemic supply of folate because of their low γ-cystathionase activity [5,6]. Recently, it was reported that systemic administration of folic acid improves axonal regeneration in injured mice through an epigenetic mechanism involving DNA methylation [7]. The biological plausibility of this mechanism is strengthened by the fact that folates supply methyl groups to the universal methyl donor S-adenosylmethionine (AdoMet). Folic acid may therefore be useful in clinical interventions to promote healing in neural tissues.

Recovery from injury in the CNS involves neural stem cells (NSCs), which are self-renewing, multipotent cells that generate neurons and glial cells [8,9]. Our previous studies showed that folic acid supplementation stimulates proliferation of rat NSCs in vitro [10–12]. Folic acid, which is used often for supplementation, is reduced and methylated to become the 5-methyltetrahydrofolate (5-MTHF) that is transported across the blood–brain barrier. It is important to determine the dose-dependent effects of folic acid and 5-MTHF in NSC cultures that are potential sources of transplantable cells for therapies for neurodegenerative diseases because large-scale culture and amplification of NSCs have not yet been achieved. Additionally, the present study tests the hypothesis that folate promotes NSC proliferation by activating DNA methyltransferase (DNMT).

2. Materials and methods

2.1. Cell culture

The Tianjin Medical University Animal Ethics Committee approved all experimental protocols in this study. Pregnant Sprague–Dawley rats were purchased from Beijing Medical Laboratory Animal Co. Ltd. (Beijing, China). Every experiment was independent because different rats were used for each experiment. Tissue from the
hippocampus region was isolated from neonatal Sprague–Dawley rats (postnatal less than 24 h, PS) and washed three times with Dulbecco’s modified Eagle’s medium (DMEM). The tissue was cut into small pieces and then dissociated by incubation with 0.25% pancrezyme and 0.02% EDTA. This step was followed by agitation, centrifugation and resuspension of the cells in DMEM and nutrient mixture F-12 Ham (F12) (1:1) (Gibco, Carlsbad, CA, USA) that was supplemented with 2% B27 supplement, 20 ng/ml epidermal growth factor (EGF), 20 ng/ml basic fibroblast growth factor (bFGF), 2 mmol/l l-glutamine, 100 U/ml penicillin and phyto觃men (Gibco, Carlsbad, USA). A custom-made powered DMEM, identical to a standard DMEM but without folic acid, was purchased from Gibco-BRL (Paisley, UK). The resulting cell suspension was aliquoted, at a density of 1×10^6 cells/ml, into T25 culture flasks (Corning Inc., Corning, NY, USA) and grown at 37°C in a humidified atmosphere containing 95% air 5% CO2.

The NSCs were exposed to the indicated concentrations of folic acid or 5-MTHF (0–200 μmol/l) for 24, 48 or 72 h. To evaluate the role of DNMT, cells were exposed for 48 h to medium containing 150 μmol/l of DNMT inhibitor zebularine and the indicated concentrations of either folic acid or 5-MTHF. The number of cells was counted after dissociation and prior to seeding into the plate.

2.4. DNMT activity

For fluorescent staining, cells were fixed in 4% paraformaldehyde for 20 min at room temperature, then washed with phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. After blocking with 10% goat serum in PBS for 1 h, cells were incubated with the primary antibodies [anti-Sox2, 1:100, Abcam; anti-β-tubulin, 1:30, Abcam; anti-glial fibrillary acidic protein (GFAP), 1:100, Millipore] overnight at 4°C. After repeated rinses in PBS, appropriate secondary antibodies (fluorescein isothiocyanate, 1:100 and TRITC, 1:100, Jackson Immunoresearch, West Grove, PA, USA) and resuspension of the cells in DMEM/F12 with bFGF and EGF. By the end of 1 week in culture, NSCs were dissociated and cultured in serum-free medium composed of DMEM/F12 with bFGF and EGF. Cells were counterstained with anti-Sox2 antibody (Fig. 1B) and merged (D). (E) Cells were counterstained for β-tubulin-III (green, 200×). (F) Cells were counterstained for GFAP (red, 200×).

Fig. 1. Identification of NSCs. (A) Photomicrographs of NSC neurospheres (100×). Cells were counterstained for Sox2 (B, green, 100×)/DAPI (C, blue, 100×) and merged (D). (E) Cells were counterstained for β-tubulin-III (green, 200×). (F) Cells were counterstained for GFAP (red, 200×).

Nuclear extracts were isolated using the nuclear extraction kit (Merck KGaA, Darmstadt, Germany). DNMT activity was measured in these extracts by using Active Motif DNA Methyltransferase Activity/Inhibition Assay Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s instructions. With DNMT activity assay, the same amount of cells from each sample was analyzed. A lot specific standard curve was used for measuring the DNMT activity. Density (OD) was measured on a microplate reader at 450 nm, and DNMT activity (OD/h/mg) was calculated according to the following formula:

DNMT activity (OD/h/mg) = (Average sample OD−average blank OD) × 1000

\[*=\text{Protein amount added into the reaction.}

\[**=\text{Incubation time used for the reaction.}

2.5. Statistical analysis

Data were expressed as mean±standard error for each treatment group based on four or five independent experiments. One-way analysis of variance (ANOVA) or factorial ANOVA was used to compare the differences between control and treatment groups. When significant, ANOVA was followed by a post hoc test (Tukey’s honestly significant difference test or Dunnet’s test). Profile analysis was used to compare the differences between folic acid and 5-MTHF. All analyses were performed using statistical software SPSS (Chicago, IL, USA) 16.0. Significance was set at α≤0.05.

3. Results

3.1. Cell identification

Tissues from the hippocampus of neonatal rats were isolated, dissociated and cultured in serum-free medium composed of DMEM/F12 with bFGF and EGF. By the end of 1 week in culture, NSCs were observed to have aggregated as spheroids, called neurospheres, which floated in suspension. Viable neurospheres were semitransparent phase contrast bright, with many of the cells on the outer surface displaying micropipes (Fig. 1A). Sox2 is essential to maintain self-renewal of undifferentiated stem cells and widely used to identify multipotential neural stem/progenitor cells in vitro. Immunofluorescence staining showed nearly all the cells of neurospheres reacted positively with anti-Sox2 antibody (Fig. 1B–D). When kept in the...
serum-free medium with bFGF and EGF, NSCs continued proliferating for several weeks. To differentiate NSCs, 5% fetal bovine serum was added to the medium without bFGF and EGF. Subsequently, sub-populations of cells exhibited immunoreactivity to β-tubulin-III and GFAP (Fig. 1E and F), which are the commonly used markers for neurons and astrocytes, respectively. These results showed that the isolated cells were NSCs, which could self-renew or differentiate into neurons and astrocytes.

3.2. Stimulation of NSC proliferation by folate is dose and time dependent

BrdU incorporation was measured in NSCs that had been incubated with 1.5, 10 and 20 μmol/L folic acid or incubated with 10 and 20 μmol/L 5-MTHF for 48 h (Fig. 2). The cells were exposed to BrdU, followed by dual staining for Sox2 and BrdU. Compared to the deficient group, 10 and 20 μmol/L of either folic acid or 5-MTHF

![Fig. 2. Immunocytochemical analysis at cells in neurospheres (phase contrast photomicrographs). (A–C) NSCs after incubation in folate deficiency medium containing 1.5 μmol/L folic acid (FA) for 48 h. (D–F) NSCs after incubation in medium containing 10 μmol/L FA for 48 h. (G–I) NSCs after incubation in medium containing 10 μmol/L 5-MTHF for 48 h. (J–L) NSCs after incubation in medium containing 20 μmol/L FA for 48 h. (M–O) NSCs after incubation in medium containing 20 μmol/L 5-MTHF for 48 h. The left panels represent NSCs stained for Sox2 (green), the middle panels represent proliferating cells stained for BrdU (red), and the right panels represent their merged images (yellow). All images were taken at the same magnification of 100×.](image-url)
increased the numbers of neurospheres. And then compared to the deficient group (78.6%±4.1%), 10 and 20 μmol/L of either folate (85.4%±3.8% and 89.7%±4.4%) or 5-MTHF (85.7%±5.1% and 90.2%±2.7%) increased the proliferating (BrdU-positive) cells. This finding demonstrates that folate dose-dependently stimulates NSC proliferation (Fig. 2).

Fig. 3. Folate increased NSC proliferation in a dose- and time-dependent manner. (A) The effect of 5-MTHF (0–200 μmol/L) on NSC proliferation after 24, 48 and 72 h of exposure was assessed by MTT assay. (B) The effect of FA (1.5–200 μmol/L) on NSC proliferation after 24, 48 and 72 h of exposure was assessed by MTT assay. Panels (C), (D) and (E) compare the effects of 5-MTHF and FA on NSC proliferation for 24, 48 and 72 h, respectively. The plotted values are mean ± standard error values from five independent experiments, with triplicate determinations in each experiment. Statistical analysis was performed using the factorial ANOVA. *NSC proliferation was greater at 5-MTHF (10–200 μmol/L) or FA (10–200 μmol/L) compared to deficient group (P<0.05).

Fig. 4. FA and 5-MTHF increased NSC proliferation with similar time courses. (A) The growth curve of NSCs treated with 5-MTHF (0–200 μmol/L) for 24, 48 and 72 h assessed by MTT assay. (B) The growth curve of NSCs treated with of FA (1.5–200 μmol/L) for 24, 48 and 72 h assessed by MTT assay. Panels (C) and (D) compare the effects of 5-MTHF and FA at 10 and 20 μmol/L, respectively. The plotted values are mean ± standard error values from five independent experiments, with triplicate determinations in each experiment.
The effects of folic acid and 5-MTHF on the proliferation of NSCs were evaluated further by MTT assay for 24, 48 or 72 h (Figs. 3–4), and the results were confirmed by cell counting (data not shown). Both forms of folate showed a dose- and time-dependent proliferative effect. By factorial ANOVA analysis, the data showed that NSC proliferation was greater at folic acid concentrations of 10–20 μmol/L compared to the deficient group (P<.05) and that the greatest effect occurred with exposure to 20 μmol/L folic acid for 72 h. Within the folic acid treatment groups, 20 μmol/L yielded the greatest growth increases over the deficient group, being 172%±8%, 177%±10% and 144%±11%, respectively, for 24, 48 and 72 h. This indicated that 20 μmol/L is the optimal dose for NSC proliferation in vitro. Factorial ANOVA analysis also showed that NSC proliferation was greater at 5-MTHF concentrations of 10–200 μmol/L compared to the deficient group (P<.05) and that the greatest effect occurred with exposure to 40 μmol/L for 72 h. Profile analysis revealed that folic acid and 5-MTHF increased NSC proliferation with similar time courses (Fig. 4C and D). 5-MTHF had a greater stimulatory effect than folic acid at 24 h (P<.05) but not at 48 h and 72 h (P>.05) (Fig. 3C–E).

3.3. DNMT mediates proliferative effect of folate

Since folate supplies a methyl group to the universal methyl donor AdoMet, we hypothesized that folate promotes NSC proliferation by activating DNMT. To test this hypothesis, we measured DNMT activity in NSCs that had been incubated with various concentrations of folic acid or 5-MTHF. We observed that both forms of folate dose-dependently increased DNMT activity and NSC proliferation in parallel (Fig. 5). To further test the hypothesis, the effect of the DNMT inhibitor zebularine on folate-stimulated NSC proliferation was assessed by MTT assay. We found that there was no decrease in DNMT activity or NSC growth when zebularine was added to the NSCs treated with ≤10 μmol/L folic acid or 5-MTHF. However, zebularine decreased the effects of 20 μmol/L folic acid and 5-MTHF on both DNMT activity and NSC proliferation (Fig. 5).

4. Discussion

4.1. Stimulation of NSC proliferation by folic acid is dose and time dependent

Previously, it has been reported that folic acid stimulates proliferation of fetal and neonatal NSCs [10,11]. The present study determined the effective concentrations of folic acid and 5-MTHF and also investigated the role of DNMT.

Incubation of NSC cultures with 20 μmol/L folic acid or 40 μmol/L 5-MTHF yielded the greatest proliferation increases compared to the folate-deficient control. These concentrations are higher than the nominal folic acid concentration in commonly used cell culture media (~10 μmol/L) [14]. The relatively high requirement for folic acid or 5-MTHF in NSCs in vitro is consistent with the fact that folates are more concentrated in cerebrospinal fluid than in plasma [15–17].

In this study, BrdU labeling was used to assess proliferation of NSCs. And it can be deduced that folate increased proliferation of NSCs. The effect of folate in increasing proliferation of NSCs is very obvious. In this study, folic acid and 5-MTHF increased NSC proliferation with similar time courses. Cells can acquire folate by two separate mechanisms [18–20]. The first involves the reduced folate carrier, a transmembrane transporter with a high affinity for 5-MTHF than for folic acid. The second involves the folate receptor, a glycosphatidylinositol-anchored protein that transports folates into the cells via endocytosis and has a higher affinity for folic acid.

Folates function as enzyme cofactors that carry one-carbon units for a network of anabolic pathways known as OCM. OCM is essential for de novo purine and thymidylate synthesis and for the remethylation of homocysteine to methionine, which can be adenosylated to form the universal methyl donor AdoMet. It is possible that folate stimulates NSC proliferation by accelerating DNA and RNA synthesis.

4.2. DNMT mediates proliferative effect of folate

Folate-mediated OCM supports methylation reactions and nucleotide synthesis. As a consequence, folate deficiency leads to methyl pool depletion, and some critical genes remain unmethylated [21]. We reasoned that the stimulation of NSC proliferation by folate may be mediated by an epigenetic pathway of DNA methylation. To further test the hypothesis, the effect of the DNMT inhibitor zebularine on folate-stimulated NSC proliferation was assessed by MTT assay. Zebularine decreased the effects of 20 μmol/L folic acid and 5-MTHF on both DNMT activity and NSC proliferation. Zebularine decreased the 55.1% and 54.9% of DNMT activity and the 79.1% and 76.2% of NSC proliferation.

Fig. 5. Folate stimulated DNMT activity and DNMT-dependent cell proliferation in NSCs. (A) Cell proliferation based on MTT assay in NSCs incubated with the indicated concentrations of 5-MTHF (0–20 μmol/L) or FA (1.5–20 μmol/L), with or without the DNMT inhibitor zebularine (Zeb). (B) DNMT activity. The plotted values are mean±standard error values from four independent experiments, with triplicate determinations in each experiment. Statistical analysis was performed using the one-way ANOVA. *P<.05.
activity influences the NSC proliferation. This means that DNMTs contribute to the alterations in cell proliferation but may not be the driver (not sure yet). In the present study, we also demonstrated that folate stimulates proliferation of NSC cells. So, the NSC proliferation is affected by two ways in this study: folate and DNMT. Folate is the donor of methylation. In the present study, we found that folate-induced growth of NSCs was associated with DNMT activation and was attenuated by a DNMT inhibitor.

Previous research found that folic acid regulates the expression of Notch1, Hes1 and Mash1 in NSCs [11]. Notch1 is a signaling receptor that influences cell fate, proliferation and survival [22]. Hes1 regulates progenitor cell proliferation and maintenance of stem cell phenotype, while Neurog2 is critical for sensory neurogenesis [23,24]. Thus, it was hypothesized that there would be a mediator between folate and its effectors Notch1, Hes1, Mash1 and Neurog2.

In the present study, DNMT activity was found to increase with folic acid concentration. Furthermore, when the cells were treated with zebularine, the DNMT activity and NSC proliferation responses to the optimal dose of folic acid were attenuated. Because zebularine forms a covalent complex with DNMT and thereby inhibits DNMT activity, it can be deduced that folate promotes NSC proliferation by activating DNMT, which may in turn regulate Notch1, Hes1, Mash1 and Neurog2.

Folic acid increases the expression of demethylase enzyme KDM6B, which in turn decreases H3K27 methylation of Hes1 and Neurog2 promoters, thereby affecting gene transcription [25,26]. In addition, KDM6B expression may be regulated by miRNAs [25], and since many miRNAs depend on DNA methylation [27–29], KDM6B-targeting miRNAs may also be under the control of DNMT. Therefore, folic acid may stimulate proliferation in NSCs by activating DNMT, which in turn affects KDM6B-targeting miRNAs, Notch1, Hes1, Mash1 and Neurog2. After determining the optimal concentration of folate for stimulation of NSC proliferation, we found that proliferation was associated with DNMT activation and was attenuated by the DNMT inhibitor zebularine. Based on these new findings and previously published evidence, we deduced a mechanism by which folate stimulates NSC proliferation (Fig. 6).

In summary, this study determined the optimal concentrations of folic acid and 5-MTHF for large-scale culturing and amplification of NSCs in vitro. Additionally, we found that folate-induced proliferation of NSCs was associated with DNMT activation and was attenuated by the DNMT inhibitor zebularine. These results suggest that the effect of folate on NSCs is caused at least in part by epigenetic regulation through DNA methylation. We believe that this is one of the mechanisms of NSCs proliferation by folate. Based on these new findings and previously published evidence, we deduced a mechanism by which folate stimulates NSC proliferation. These novel findings shed new light on the mechanism of action of folate in CNS development and suggest new approaches for the prevention and treatment of neurodegenerative diseases.

Acknowledgments

The authors’ responsibilities were as follows — G.H.: developed and designed the concept; W.L., M.Y., S.L., H.L. and Y.G.: designed and conducted the research; W.L.: performed the statistical analysis; W.L., M.Y. and J.X.W.: drafted the manuscript; W.L., M.Y. and G.H.: had primary responsibility for the final content. All authors read and approved the final manuscript. None of the authors declared a conflict of interest.

References

[11] Zhang YM, Liu H, Gong GX, Tian ZH, Ren DL, Wilson JX. Folic acid increases the expression of demethylase enzyme KDM6B, in turn decreases H3K27 methylation of Hes1 and Neurog2 promoters, thereby affecting gene transcription [25,26]. In addition, KDM6B expression may be regulated by miRNAs [25], and since many miRNAs depend on DNA methylation [27–29], KDM6B-targeting miRNAs may also be under the control of DNMT. Therefore, folic acid may stimulate proliferation in NSCs by activating DNMT, which in turn affects KDM6B-targeting miRNAs, Notch1, Hes1, Mash1 and Neurog2. After determining the optimal concentration of folate for stimulation of NSC proliferation, we found that proliferation was associated with DNMT activation and was attenuated by the DNMT inhibitor zebularine. Based on these new findings and previously published evidence, we deduced a mechanism by which folate stimulates NSC proliferation (Fig. 6).

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