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Folate deficiency-induced oxidative stress contributes to neuropathy in young and aged zebrafish — Implication in neural tube defects and Alzheimer's diseases

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ABSTRACT

Folate is a nutrient essential for the development, function and regeneration of nervous systems. Folate deficiency has been linked to many neurological disorders including neural tube defects in fetus and Alzheimer's diseases in the elderly. However, the etiology underlying these folate deficiency-associated diseases is not completely understood. In this study, zebrafish transgenic lines with timing and duration-controllable folate deficiency were developed by ectopically overexpressing a recombinant EGFP-γ-glutamyl hydrolase (γGH). Impeded neural crest cell migration was observed in the transgenic embryos when folate deficiency was induced in early stages, leading to defective neural tube closure and hematopoiesis. Adding reduced folate or N-acetylcysteine reversed the phenotypic anomalies, supporting the causal link between the increased oxidative stress and the folate deficiency-induced abnormalities. When folate deficiency was induced in aged fish accumulation of beta-amyloid and phosphorylated Tau protein were found in the fish brain cryo-sections. Increased autophagy and accumulation of acidic autolysosome were apparent in folate deficient neuroblastoma cells, which were reversed by reduced folate or N-acetylcysteine supplementation. Decreased expression of cathepsin B, a lysosomal protease, was also observed in cells and tissue with folate deficiency. We concluded that folate deficiency-induced oxidative stress contributed to the folate deficiency-associated neuropathogenesis in both early and late stages of life.

Introduction

Folate (folic acid, vitamin B9) is essential for health from early life to old age (McNulty et al., 2012). Folate pathway is vital for the development, regeneration and function of nervous systems (Iskandar et al., 2010). Folate deficiency and impaired folate pathway have been linked to many diseases, especially neurological disorders (Stover, 2009). Currently, the causative mechanisms underlying most of these folate-associated pathogeneses are not completely understood.

Folate is the major intracellular one-carbon carrier. A one-carbon unit of three different oxidative states: methanol, formaldehyde or formate, is attached to the pteridine ring of folate, yielding different folate adducts. In cells, folate is polyglutamylated to form biologically active folylpolyglutamates (Cossins, 1984). The inter-conversion between different folate adducts occurs when these folates provide their one-carbon units for generating molecules required for myriads of biological processes. The inter-conversion also occurs via one-carbon metabolism (OCM) in which several redox and synthetic reactions are catalyzed by folate enzymes (Fig. 1). Folate is vital for rapidly growing tissues and proliferating cells, such as fetus and cancer, because it participates in the biosynthesis of nucleotides, amino acid, some vitamins and neurotransmitters. Folate is crucial for epigenetic control because it provides the one-carbon unit required for S-adenosylmethionine (SAM) biosynthesis. SAM is the primary methyl donor for DNA/RNA, protein and lipid methylation, endowing folate the potential to modulate gene activity simply via dietary intervention. An individual's folate status in young life may affect "fetal programming" by modulating embryonic gene activity and cause developmental adaptations and permanent alterations that lead to predisposed risks to diseases in the affected individual's adult life or even pass down generations (Anway et al., 2005; Ciappio et al., 2011; Grissom et al., 2013).

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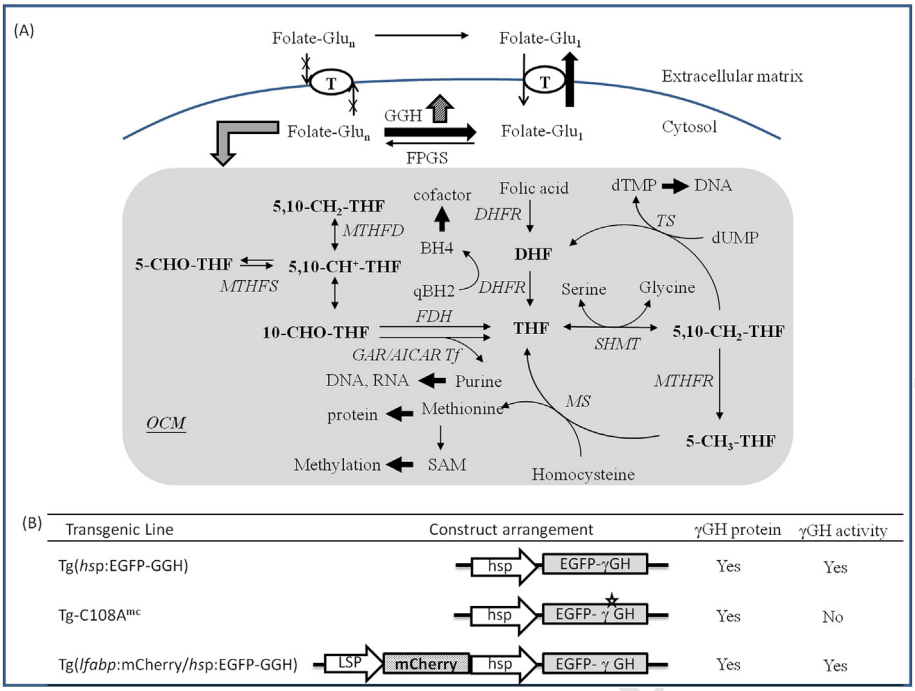

Fig. 1. Depicted mechanism and clones for generating zebrafish transgenic lines with heat-shock inducible folate deficiency. (A) Over-expressed γGH increases the ratio between monoglutamylfolates and polyglutamylfolates, leading to facilitated folate exportation and decreased intracellular folate. "T" represents the transporters embedded in the cell membrane and responsible for transporting folate in and out of the cells. Reactions involving folate coenzymes and enzymes of one-carbon metabolism (OCM) are responsible for the biosynthesis of purine, thymidylate and SAM. (B) The constructs for generating inducible folate deficiency in transgenic fish encompassing an EGFP-γGH fusion coding sequence driven by heat-shock promoter. The construct containing the C108A point mutation in the γGH coding sequence (Tg-C108A^{mc}) was created to serve as a γGH functional control. An additional mCherry coding sequence driven by a liver specific promoter was included in the third construct for creating the transgenic line Tg(lfabp:mCherry/hsp:EGFP-γGH), which will also express mCherry specifically in liver. The following enzyme abbreviations are: SHMT, serine hydroxymethyltransferase; FDH, 10-formyltetrahydrofolate dehydrogenase; DHFR, dihydrofolate reductase; MTFHS; methynyltetrahydrofolate synthase; MTHFD, methylenetetrahydrofolate dehydrogenase; TS, thymidylate synthase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; GAR/AICAR Tf, glycinamide ribonucleotide ttransformylase and aminoimidazolecarboxamide ribotide transformylase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

The growing awareness of the pathogenesis associated with folate deficiency has drastically increased the public demand for folic acid supplementation. Besides folate fortification, ample amounts of folic acid are often ingested by pregnant mother and general population as a daily supplement. Beneficial effects of folate fortification and supplementation in preventing neural tube defects (NTD) have been well-documented. However, detrimental effects caused by unmetabolized folic acid and supraphysiological folate also appear, leading to a vigorous debate on mandatory folate fortification and supplementation among researchers (Moore et al., 2013; Osterhues et al., 2013; Strickland et al., 2013). This controversy reveals an urgent need for the study and proper tool to understand the mechanisms underlying folate associated pathogenesis.

Zebrafish is a prominent model vertebrate in various biological disciplines. Possessing the advantages of "in vitro convenience" and "in vivo complexity", zebrafish is ideal to complement rodent for a "real-time", "dynamical" and "high-throughput" observation. The similarity between neurulation in zebrafish and mammals supports the properness of using zebrafish for understanding neural tube development and related pathogenesis (Lowery and Sive, 2004). The availability of both larva and adult duality enable investigation of a wide-spectrum on neuropathogenesis throughout ontogenesis. However, the study about folate-mediated OCM of/with zebrafish has been limited mostly due to lacking a proper protocol to induce folate deficiency in zebrafish. Owing to fish feeding habits and living environment, creating a folate-deficient condition in zebrafish (and other aquatic organisms) is intrinsically difficult. It is almost impossible to feed the fish with a "folate-free" diet or to estimate the quantity of ingested folate since fish eat baby shrimp, algae and plankton besides the provided food. The strategy of adding folate antagonists, such as methotrexate, was likely to cause folate "imbalance", instead of "deficiency" (Kao et al., 2013). The challenges hence arise for researcher to develop an assessable folate deficient model with zebrafish.

In order to understand how folate affects nervous system in different stages of life, we established a zebrafish folate deficient model by over-expressing a fusion of enhanced green fluorescent protein (EGFP) with γ-glutamylhydrolase (γGH) controlled by a heat-shock promoter (hsp). γGH converts polyglutamyl-folates to monoglutamyl-folates. Past studies had shown that only the monoglutamate forms of folate adducts cross the cell membrane and that retention of folates in the cell is accomplished by polyglutamylation by the enzyme folylpolyglutamate synthetase (FPGS in Fig. 1) (Liu and Ulrich, 2009). Therefore, the overexpressed γGH would facilitate folate exportation, leading to diminished intracellular folate pools. The use of the heat-shock promoter allowed the induction of folate-deficiency at desired stages with controllable extent and duration. Green fluorescence allowed the estimation for the intensity of γGH expression and folate deficiency. The anatomical and pathological characteristics of these folate deficient transgenic embryos and aged fish were examined. The displayed characteristics evidenced the occurrence of folate deficiency in these transgenic fish. The mechanisms involved in the folate deficiency-induced neuropathy were also investigated.

Materials and methods

Material

All reduced folates were gifts from Dr. Moser (Merck Eprova AG, Switzerland). The *Lactobacillus casei* for measuring total folate was obtained from Food Industry Research and Development Institute (Hsin-Chu, Taiwan). The plasmid encoding GFP-LC3 was a gift originally from Dr. Tamotsu Yoshimori and Dr. Noboru Mizushima/University of

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