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Spontaneous neural tube defects in splotch mice supplemented with selected micronutrients

Bogdan J. Wlodarczyk [a,*], Louisa S. Tang [a], Aleata Triplett [b], Frank Aleman [b], Richard H. Finnell [a,b]

[a] *Center for Environmental and Genetic Medicine, Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, TX 77030, USA*

[b] *Center for Human Molecular Genetics, Department of Pediatrics, University of Nebraska Medical Center, Omaha, NE 68198-5455, USA*

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Abstract

Splotch (Sp/Sp) mice homozygous for a mutation in the Pax3 gene inevitably present with neural tube defects (NTDs), along with other associated congenital anomalies. The affected mutant embryos usually die by gestation days (E) 12–13. In the present study, the effect of modifier genes from a new genetic background (CXL-Sp) and periconceptional supplementation with selected micronutrients (folic acid, 5-formyltetrahydrofolate, 5-methyltetrahydrofolate, methionine, myoinositol, thiamine, thymidine, and α-tocopherol) was determined with respect to the incidence of NTDs. In order to explore how different exposure parameters (time, dose, and route of compound administration) modulate the beneficial effects of micronutrient supplementation, female mice received either short- or long-term nutrient supplements via enteral or parenteral routes. Embryos were collected on E12.5 and examined for the presence of anterior or posterior NTDs. Additionally, whole mount in situ hybridization studies were conducted in order to reveal/confirm normal expression patterns of the Pax3 gene during neurulation in the wild-type and Sp/Sp homozygous mutant mouse embryos utilized in this study. A strong Pax3 signal was demonstrated in CXL-Sp embryos during neural tube closure (E9.5 to E10.5). The intensity and spatial pattern of expression were similar to other Splotch mutant mice. Of all the micronutrients tested, only supplementation with folic acid or 5-methyltetrahydrofolate rescued the normal phenotype in Sp/Sp embryos. When the folate supplementation dose was increased to 200 mg/kg in the diet, the incidence of rescued splotch homozygotes reached 30%; however, this was accompanied by six-fold increased resorption rate.

Keywords: Splotch; Neural tube defects; Folic acid; 5-Formyltetrahydrofolate; 5-Methyltetrahydrofolate; Methionine; Myoinositol; Thiamine; Thymidine; Tocopherol; Pax3; Gene expression

Introduction

Neural tube defects (NTDs), collectively among the most common of all human congenital malformations, are the result of defective closure of the neural tube during early gestation. The prevalence of these defects in the human population varies by country, geographical region, and ethnicity, ranging from 1 to 60 per 10,000 births (ICBDMS, 2000; Moore et al., 1997; NBDPN, 1997). The most common forms of NTDs are anencephaly, which is a malformation characterized by the absence of cranial vault with the brain missing or greatly reduced, and spina bifida, which includes malformations of the spinal column characterized by herniation or exposure of the spinal cord through an incompletely closed spine. Numerous epidemiological and experimental studies indicate that the etiology of NTDs is multifactorial, with both genetic and environmental factors contributing (Campbell et al., 1986; Copp and Bernfield, 1994; Finnell et al., 2000). Although the mechanism responsible for NTDs has yet to be defined, the incidence and severity of these malformations more likely are determined by the interaction of genetic predisposing factors with participating environmental agents. Extensive epidemiological studies pioneered by Smithells et al. (1980) and continued by other investigators in many countries revealed that folic acid supplementation could greatly reduce the incidence of NTDs (Milunsky et al., 1989; Wald et al., 1991; Czeizel and Dudas, 1992). It has been estimated that approximately 50–70% of NTD occurrences, as well as recurrent NTDs, can be prevented if women receive

* Corresponding author. Fax: +1 713 677 7784.
E-mail address: bwlodarczyk@ibt.tamhsc.edu (B.J. Wlodarczyk).

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supplementation with folate during the periconceptional period (Acuna et al., 1999).

In order to elucidate the mechanisms underlying the disturbances of neurulation, as well as the modulatory effect of folic acid and/or other micronutrients on the process of neurulation, different experimental models have been extensively exploited. The models most often used are murine mutants which exhibit NTD phenotypes. These include curly-tail (ct), splotch (Sp), crooked-tail (Cd), loop-tail (Lp), axial defect (Axd), snubnose (sno), SELH, and other mouse mutants (Greene and Copp, 2005). Recently, additional murine NTD models became available with the advent of genetically modified mice. Such animals have been constructed by inactivating genes involved in the process of neurulation, including Folbp1, Cart-1, RFC1, p53, HES-1, Cited2, and others (Fleming et al., 1997; Piedrahita et al., 1999; Juriloff and Harris, 2000; Spiegelstein et al., 2004).

Splotch mutant mice have long been used by investigators as a model system for the study of NTDs. These mice carry semi-dominant mutations in the transcription factor Pax3, which arose spontaneously in the C57BL/6J mouse strain (Russell, 1947). In embryos that are homozygous (Sp/Sp), the splotch mutation results in several malformations including those of the nervous system (spina bifida with or without exencephaly, reduced or absent spinal ganglia, deficiency of Schwann cells), cardiovascular system (cardiac malformations involving partial or complete failure of septation of the outflow tract, so called conotruncal heart defects), skin (absence of melanocytes), and muscles (reduced axial muscles along a rostro-caudal gradient and slower development of limb muscles) (Auerbach, 1954; Franz, 1989, 1990; Franz et al., 1993; Conway et al., 1997; Epstein et al., 2000). The homozygous Sp/Sp embryos die in utero by E14. Splotch heterozygotes (Sp/+) are viable and exhibit a pigmentation defect present as a white belly spot, sometimes extending to the limbs and tail. The white spots result from the failure of neural crest cell derived melanocytes migrating into these regions. Several alleles of splotch have been identified (Sp, Sp^{J}, Sp^{2J}, Sp^{3J}, Sp^{d}, Sp^{r}, Sp^{1H}, Sp^{2H}, Sp^{4H}), and all of them harbor mutations in the Pax3 gene, which has been mapped to mouse chromosome 1 (Dickie, 1964; Epstein et al., 1991; Goulding and Paquette, 1994).

Mutations in the human PAX3 gene (homolog of the mouse Pax3 gene) are also associated with several congenital defects. A sequence of malformations secondary to PAX3 mutations has been defined as Waardenburg Syndrome (WS) types I and III (Waardenburg, 1951; Tassabehji et al., 1993). This syndrome is a dominant disorder characterized by pigmentation disturbances and cochlear deafness; however, investigators have also reported a wide range of other phenotypes (Klein, 1983; Read and Newton, 1997). Type III is the most severe form of WS, which include upper limb abnormalities in addition to the more classical defects associated with this syndrome. Some WS cases are associated with facial clefts, NTDs, microcephaly, and mental retardation. The prevalence of this disorder is estimated at 1/42,000 births in the human population.

Similar phenotypic features are shared between both human and mice bearing abnormalities in their PAX3/Pax3 genes. Given the high (98%) sequence similarity of this gene product between these two species, there is every suggestion that the splotch mouse can serve as a good model for studying defects caused by mutations in PAX3 gene. In an effort to elucidate the mechanism underlying the etiology of NTDs, and to further efforts to help prevent the occurrence of these defects, numerous studies have been conducted on mutant mice further demonstrating that maternal supplementation with micronutrients have a modulatory effect on the incidence of NTDs in developing embryos. Furthermore, there are some substances (e.g., folic acid, methionine, inositol, thymidine) that can significantly reduce the number of NTD affected pups (Essien, 1992; Zhao et al., 1996; Greene and Copp, 1997; Carter et al., 1999; Shin and Shiota, 1999). There is also evidence from these studies that longer supplementation periods can greatly enhance the beneficial effect (Nosel and Klein, 1992).

In the present study, we examined the effect of a new genetic background as well as supplementation with selected micronutrients (folic acid, 5-formyltetrahydrofolate, 5-methyltetrahydrofolate, methionine, myoinositol, thiamine, thymidine, α-tocopherol) has on the incidence of NTDs in CXL-Sp embryos. In order to explore how different exposure parameters (time, dose, and route of compound administration) modulate the beneficial supplementation effect, female mice received either short- or long-term nutrient supplements either via an enteral or parenteral route. Additionally, whole mount in situ hybridization studies were conducted in order to reveal/confirm normal expression pattern of Pax3 gene during neurulation in wild type and Sp/Sp homozygous mutant mouse embryos utilized in this study.

Methods

Experimental animals

In this study, we used the inbred CXL-Sp mouse strain, which originated from crosses between the C57BL/J6-Sp and LM/Bc/Fnn inbred strains (Gefrides et al., 2002). The fragment of CLX-Sp Pax3 gene covering 3′ extremity of intron 3 and the 5′ extremity of exon 4 was sequenced, and the splotch mutation was confirmed to be identical to the spontaneous Sp mutation described by Epstein et al. (1993). In order to explore the effect of micronutrient supplementation on homozygous (Sp/Sp) CXL-Sp embryos, we mated heterozygous (Sp/+) females with heterozygous (Sp/+) males. The mice were maintained on a 12-h light/dark cycle in the Vivarium of the Institute of Biosciences and Technology, Houston, TX. Up to five females were housed in clear polycarbonate cage and were allowed free access to water and food. Virgin females, 50–70 days of age, were mated overnight with males and examined for the presence of vaginal plugs the following morning. The onset of gestation was set at 10:00 p.m. of the previous night, the midpoint of the dark cycle (Snell et al., 1948).

Virgin females were randomly assigned into one of twelve experimental groups (Table 1). Solutions for the animal treatments were prepared ex tempore. All supplements except vitamin E were dissolved in sterile water for injection (USP, Abbott Laboratories, Chicago, IL). α-Tocopherol was prepared in 25% water solution of Cremophore EL (Fluka Biochemica). Each experimental group consisted of 8–11 females, except groups III and IV (both treated with 5-FTHF), which consisted of 16 and 21 females, respectively. Our previous study (Piedrahita et al., 1999; Spiegelstein et al., 2004) indicated that folinic acid was very effective in protecting nullizygous Folbp1 knockout mouse embryos from NTDs.

Treatment rationale

In order to mimic the way pregnant women are exposed to folate and other micronutrient (pills and supplemented food), we chose to treat most of the