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Alx3-deficient mice exhibit folic acid-resistant craniofacial midline and neural tube closure defects

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ABSTRACT

Neural tube closure defects are among the most frequent congenital malformations in humans. Supplemental maternal intake of folic acid before and during pregnancy reduces their incidence significantly, but the mechanism underlying this preventive effect is unknown. As a number of genes that cause neural tube closure defects encode transcriptional regulators in mice, one possibility is that folic acid could induce the expression of transcription factors to compensate for the primary genetic defect. We report that folic acid is required in mouse embryos for the specific expression of the homeodomain gene *Alx3* in the head mesenchyme, an important tissue for cranial neural tube closure. *Alx3*-deficient mice exhibit increased failure of cranial neural tube closure and increased cell death in the craniofacial region, two effects that are also observed in wild type embryos developing in the absence of folic acid. Folic acid cannot prevent these defects in *Alx3*-deficient embryos, indicating that one mechanism of folic acid action is through induced expression of Alx3. Thus, *Alx3* emerges as a candidate gene for human neural tube defects and reveals the existence of induced transcription factor gene expression as a previously unknown mechanism by which folic acid prevents neural tube closure defects.

Introduction

The formation of the neural tube during the early stages of neural development, known as neurulation, occurs by apposition and fusion of the neural folds, which arise and elevate from both sides of the neural plate. Closure of the neural tube during neurulation is a complex process that is initiated at different contact points both in mice and in humans (Copp et al., 2003; De Marco et al., 2006; O'Rahilly and Muller, 2002). When this process fails, the neural tube remains partially open and congenital neural tube defects (NTD) develop. There are different types of NTD depending on the location of the defect, including acrania and anencephaly at the cranial level, and spina bifida at the spinal level. In humans, NTD represent an important group of congenital disorders with a relatively high incidence.

The molecular bases of NTD in humans are largely unknown. Environmental factors appear to be important, but accumulating evidence indicates the existence of a strong genetic component. In recent years, the development of mouse models with NTD has led to the identification of a relatively large number of genes that are important for neural tube closure (Copp et al., 2003; Harris and Juriloff, 2007). Although these studies provide an impressive list of candidate genes possibly involved in abnormal neurulation in humans, with few exceptions (Kibar et al., 2007b) clinical studies have failed to establish etiological associations between NTD and mutations in any of the homologous human genes (Kibar et al., 2007a).

It is now well established that folic acid supplementation to women during their child-bearing age can decrease the incidence of NTD up to 70% (Czeizel and Dudas, 1992; Wald et al., 1991), but the mechanism of action of this vitamin on the prevention of defective neural tube closure is not known (Blom et al., 2006). In mouse models, disruption of the genes encoding folic acid binding protein 1 (Folbp1) or reduced folate carrier 1 (RFC1) causes NTD, (Gelineau-van Waes et al., 2008; Piedrahita et al., 1999). In both cases, maternal supplementation with folic acid rescues the defect, underscoring the importance of efficient transport of folates into cells for normal development. Interestingly, disruption of the gene encoding methylenetetrahydrofolate reductase, a key enzyme in folate metabolism, does not result in the appearance of NTD (Chen et al., 2001). In humans, it has been proposed that the etiological basis of NTD responds to a multifactorial model in which both genetic and environmental factors interact in a complex manner (Harris and Juriloff, 2007; Zohn and Sarkar, 2008).

In some mouse models, the disruption of genes encoding transcriptional regulators such as Pax3, Cart1 and Cited2 results in the development of NTD that can be rescued by maternal folate

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supplementation (Fleming and Copp, 1998; Martinez-Barbera et al., 2002; Zhao et al., 1996). In these cases, a defect in folate metabolism has only been documented in *Pax3*-deficient mice (Fleming and Copp, 1998). In contrast, despite the preventive effect of folic acid, the existence of a defect in folate metabolism was discarded in *Cited2*-deficient mice (Martinez-Barbera et al., 2002). In view of the emerging concept that some neural tube closure defects are unrelated to folate metabolism (Blom et al., 2006; Harris, 2001; Martinez-Barbera et al., 2002), and of the observation that a number of NTD-related genes encode proteins involved in transcriptional regulation, we argued that one possible mechanism for the protective effect of folic acid could be the induction of the expression of transcription factors that could compensate for the genetic defect. Because of the known role of homeodomain proteins in embryonic development, we sought to determine whether folic acid is able to induce the expression of genes encoding this type of transcription factors.

Materials and methods

Cell culture

RC2.E10 cells, originally derived from embryonic rat telencephalon (McManus et al., 1999), were cultured at a temperature of 33 °C in RPMI 1640 in the presence of 10% fetal bovine serum, streptomycin (10 μg/ml), penicillin (100 IU/ml) and fungizone (2.5 μg/ml). For experiments involving maintenance of cells in the absence of folic acid, cells were grown in folic acid-free RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum that had been stripped with charcoal in order to deplete endogenous folate. To compensate for the metabolic effects of folate deficiency, 200 μM glycine, 100 μM adenosine, and 10 μM thymidine (Zhao et al., 1998) (all from Sigma) were added to the culture medium. Folic acid (Sigma) was added at the indicated concentrations when required.

To determine proliferation rates in different culture conditions, cells were plated into 60 mm dishes at an initial density of 5×10^4 cells/dish. Every 24 h cells from some of the dishes were trypsinized, collected by centrifugation, and counted with a hemocytometer. Culture media of the remaining cells were changed every 2 days.

Mice

Alx3-deficient mice were provided by Dr. Frits Meijlink (Netherlands Institute for Developmental Biology, Utrecht, The Netherlands) and were maintained in a C57BL/6×FVB/N background (Beverdam et al., 2001). Genotyping was carried out by PCR using genomic DNA isolated from tail biopsies, using a REDExtract-N-Amp Tissue PCR kit (Sigma). The sequences of the oligonucleotide primers used to amplify the wild type allele are as follows: forward, 5′-CATCCCCTCTCCATGCATGTCCCC-3′; and reverse, 5′-CTAGGAGCAGGTCAGAGCAGGAAG-3′. PCR conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, after which a 5 min incubation at 72 °C followed. For amplification of the mutant alleles we used the following primers: forward, 5′-TCGAGCTGGGTAATAAGCGTTGGCAAT-3′; and reverse, 5′-AGACCAACTGGTAATGGTAGCGAC-3′. PCR conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, after which a 5 min incubation at 72 °C followed.

Mice were fed routinely a standard breeding diet (Safe-diets AO4; Panlab, Barcelona, Spain). When indicated, to test the effect of a dietary folic acid deficiency, female mice were fed a custom-made (Safe-diets AO4, Panlab) folic acid-free diet containing 1% succinyl sulfathiazole, an antibiotic that eliminates digestive tract bacteria that synthesize folates (Burgoon et al., 2002). In this case, the control diet contained both folic acid (0.5 mg/kg, which corresponds to the concentration present in the commercial standard diet) and succinyl sulfathiazole at the above mentioned concentration. Females were fed these diets for a period of at least one month before mating to males that had been fed a standard breeding diet, and during the duration of pregnancy up to E9.5. At this time, they were killed and the embryos were extracted for analysis.

Experimental protocols involving mice were approved by the institutional committee on research animal care, and meet the requirements of current Spanish and European Community legislation.

Mouse embryo culture

Mouse embryos were cultured basically as described (Cockroft, 1990). Pregnant mice were killed at 8.5 days *post coitum* by cervical dislocation and the uterus was removed and placed in Hank's balanced salt solution. Embryos were explanted from the uterus without disrupting the visceral yolk sac, and cultured for 24 h in 4 ml rat serum within glass chambers (one embryo per chamber) plugged into a rotating hollow drum (BTC Engineering, Cambridge, UK), with continuous supply of a mixture of 5% O_2, 5% CO_2 and 90% N_2. All cultures were carried out in the presence of penicillin (100 IU/ml) and streptomycin (100 μg/ml). The rotating system was maintained in a light-tight incubator at a constant temperature of 37 °C.

Rat serum to culture the embryos was prepared from blood collected from the abdominal aorta of halothane-anaesthetized animals as described (Cockroft, 1990). Initial experiments to set up the system were carried out with complete serum (see Supplemental Fig. 1). For depletion of folic acid, serum was incubated overnight with and ion exchange resin (AG1-X8, BioRad). Serum folate levels were determined by chemiluminescence as described (Achon et al., 2000). Folate concentrations before incubation with the resin were 41.37 ± 3.4 ng/ml ($n=4$), whereas after incubation they were below the detection limit of the assay (1–2 ng/ml). For embryo culture, depleted serum was supplemented with 10 μg/ml myo-inositol (Fleming and Copp, 1998; Greene and Copp, 1997), as well as with 200 μM glycine, 100 μM adenosine, and 10 μM thymidine (all from Sigma) (Zhao et al., 1998). When required, 100 μM folic acid (Sigma) was added.

RT-PCR

RC2.E10 cells growing in folic acid-free medium supplemented with 200 μM glycine, 100 μM adenosine, and 10 μM thymidine were treated with folic acid for 24 h, after which total RNA was extracted using TriReagent (Molecular Cell Center, Inc.). RNA (5 μg) was primed with poly-$(dT)_{15}$ and incubated with avian myeloblastosis virus reverse transcriptase to synthesize cDNA.

The sequences of all primers used for PCR amplifications are provided in Supplemental Table 1. For rat Alx3 (GeneBank Accession, AY488087) PCR conditions were: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, after which a 5 min incubation at 72 °C followed. Primers for PCR amplification of Otx1, Otx2, Emx1, Pdx1, Dlx2, Dlx4, Dlx5 and actin were designed based on the published sequences of the rat cDNAs (Robel et al., 1995; Schwartz et al., 2000). PCR conditions for Otx1, Otx2, Pdx1, Dlx4 and actin were identical to those of rat Alx3. PCR conditions for Dlx2 and Dlx5 were: 96 °C for 5 min, followed by 30 cycles of 96 °C for 30 s, 60 °C for 30 s, and 75 °C for 30 s, after which a 5 min incubation at 72 °C followed. In the case of Emx1, conditions were identical with the exception that annealing temperature was 65 °C.

Mouse embryos (E8.5 at the onset of culture) were cultured for 24 h in folic acid-free medium supplemented with 10 μg/ml myo-inositol, 200 μM glycine, 100 μM adenosine, and 10 μM thymidine, or in a similar medium to which folic acid was added. Total RNA was extracted from the heads and cDNA was synthesized by incubation with poly-$(dT)_{15}$ and avian myeloblastosis virus reverse transcriptase. PCR conditions for Alx3 (GeneBank Accession, NM_007441), Cart1 (GeneBank Accession, NM_172553), Twist (GeneBank Accession, NM_011658), Cited2 (GeneBank Accession, NM_010828), Alx4