Cell Death and Differentiation (2004) 11, 361–363

www.nature.com/cdd

Letter to the Editor

Folic acid rescues nitric oxide-induced neural tube closure defects

Cell Death and Differentiation (2004) **11,** 361–363. doi:10.1038/sj.cdd.4401371
Published online 19 December 2003

Dear Editor,

The importance of folic acid (FA) in the prevention of neural tube defects (NTD) involving failure of neural tube (NT) closure in the developing embryo is well established. Various forms of NTD, especially spina bifida and anencephaly, can be prevented by supplementing the diet with FA in the periconceptional period.[1,2] How FA acts to prevent NTD is still unknown. Nitric oxide (NO) had been shown to be able to induce NTD in 10.5-day rat embryos,[3] and biochemical studies showed that NO inhibits methionine synthase (MS), by interfering with the transfer of the methyl group from the methyl donor 5-methyl-tetrahydrofolate (5mTHF) to homocysteine.[4] We have shown that endogenously produced NO in the neuroepithelium, at the time of NT closure, plays a role in cell cycle progression[5] and the regulation of cell numbers in the developing NT.[6] We also found that high NO levels increase the demand for folates.[6] Here, we describe experiments that examine the direct effect of FA and NO produced by the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) on the process of NT closure in the chick embryo *ex ovo*. The data here demonstrate that exposure to NO produced by SNAP results in an open NT by inhibition of the MS reaction, thus interfering with the flow of one carbon unit through the folate pathway, possibly by vitamin B_{12} poisoning. These effects of NO can be effectively alleviated by FA or vitamin B_{12}. Thus, the findings described here may explain why FA supplementation during pregnancy prevents NT closure defects. The experimental system used here is focused on a defined 6 h window between the eight and 12 somite stage in chick embryo development. This is the time when NT closure takes place by a process that involves programmed cell death.[7] Eight somite-stage embryos were explanted as previously described,[8] and cultured for 6 h individually in DMEM conditioned agarose dishes, in the presence of the NO donor SNAP (2 mM) with or without 50 μg/ml FA. The effects of these treatments on normal NT closure in whole mount and serial NT transverse sections of 12 somite stage embryos are shown in Figure 1a. The NO levels produced by SNAP treatment in our experimental system clearly interfere with NT closure. While FA alone had no effect on NT closure, its addition to the SNAP-treated embryos alleviated the SNAP effect by a complete restoration of NT closure.

To further verify that NO is indeed the factor responsible for the NTD, experiments were performed using 4 μg/ml hemoglobin to quench NO.[9] This hemoglobin treatment in fact counteracted the SNAP effect on NT closure, resulting in normal NT closure rates (22 ± 3% open NT transverse sections, $P < 0.001$ significance by two-way ANOVA). The observation that SNAP produced NO inhibits NT closure in the chick embryo and that this inhibition is counteracted by FA raised the possibility that NO interferes with one carbon unit metabolism. This conclusion is corroborated by a recent report suggesting that NO inhibits the MS reaction, thereby blocking the flow of one carbon units through the folate pathway.[4] Our results and this report prompted us to examine the possibility that NO induction of NTD is caused by inhibition of the MS reaction. To this end, eight somite stage embryos were treated with 2 mM SNAP in the presence or absence of FA. For the last 4 h of incubation, 5-[^{14}C]m-THF was added to the embryo culture. At the end of the experiment (12 somite stage), NT closure was assessed in whole mount preparations and the embryos were processed for assaying the MS reaction by measuring the incorporation of labelled methyl groups into TCA-precipitable material. The correlation between MS activity and NT closure is demonstrated in Figure 1b. High MS activity was detected in 12 somite embryos with closed NT morphology. In this category were most of the untreated embryos and those treated with a combination of SNAP and FA. Decreased MS activity was detected in embryos treated with SNAP alone, in which open NT morphology was observed. These results suggest that normal NT closure requires normal MS activity. One carbon unit flow associated with MS activity can be used therefore as a biochemical marker of normal NT closure.

To further characterize the inhibitory effects of NO on the MS reaction and its dependence on FA availability, we assayed MS activity in dose response experiments with FA and SNAP in pooled embryo cultures. A clear concentration-dependent effect of SNAP on MS activity was observed, and that FA at a concentration of 50 μg/ml completely restored normal MS activity (Figure 1c). Since NO is known to interact with cobalamin (vitamin B_{12}),[4] a known cofactor in the methyl transfer reaction, we argued that the effect of NO on the methyl group transfer reaction may be a result of sequestration of this cofactor by NO. To test this possibility, we added 50 μg/ml of vitamin B_{12} to the MS assay system, and found that, at this concentration, vitamin B_{12} significantly restores MS activity in embryos exposed to 2 mM SNAP (Figure 1c). It is therefore tempting to speculate that the molecular mechanism by which NO inhibits the carbon flow through the folate pathway involves cobalamin poisoning by NO. This

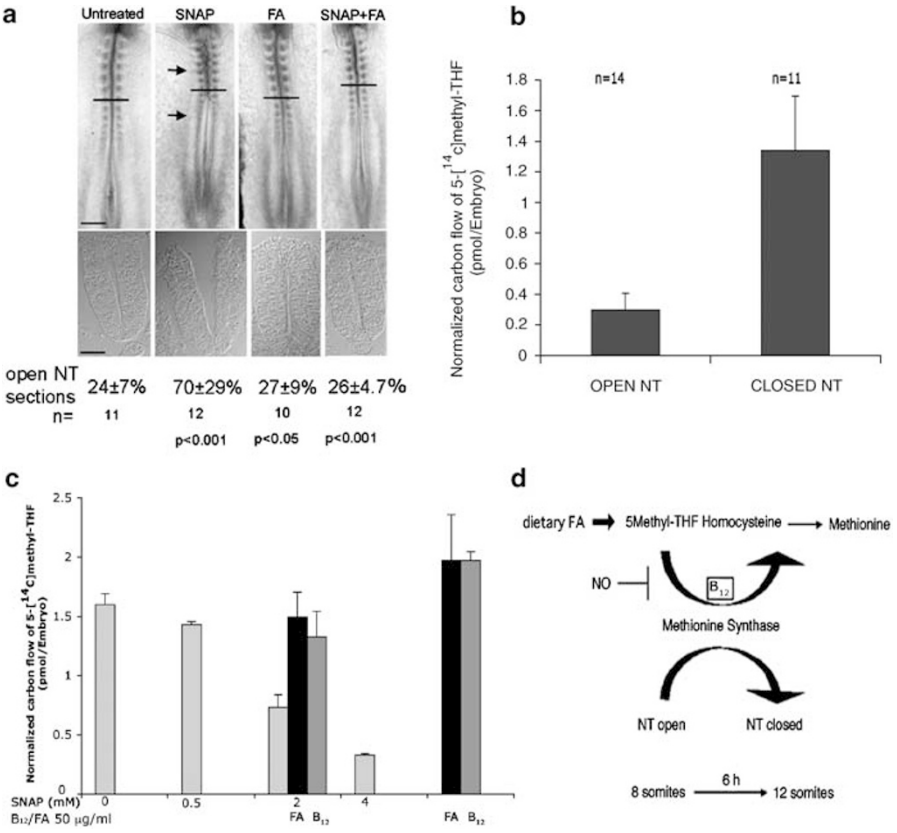

Figure 1 (**a**) Chick embryos at the eight somite stage were explanted as described before (8), and incubated in DMEM/2% agarose dish for 6 h with 2 mM SNAP, 50 μg/ml FA or a combination of SNAP and FA. The upper panel represents a dorsal view of NT morphology of 12 somite embryos from the first rostral somite down to the posterior neuropore. Note the open NT morphology along the antero-posterior axis of the SNAP-treated embryo (arrows pointing at two regions along the NT). Size bar: 400 μm. The lower panel represents differential interference contrast images of transverse cryosections, prepared as described before (7), at the level of the seventh rostral-somite region (as indicated by the horizontal lines in the upper panel). These micrographs show the difference in NT closure status. Size bar: 40 μm. At the bottom, the calculated percentage of open NT sections is presented, based on the analysis of six independent experiments showing a mean $\pm$ S.D. value. n = number of embryos analyzed, P = significance calculated by two-way ANOVA. (**b**) Explanted embryos were treated as described above and supplemented with 0.2 μCi of 5-[^{14}C] methyl-THF (57 μCi/μmol, Amersham Biosciences) the last 4 h of the incubation period, to assess the methyl transfer through the MS reaction into TCA-precipitated material, as previously described (4). NT closure was visualized in whole mount preparations as described in (**a**). Radiolabeled embryo extracts were made from individual cultured embryos as follows: embryos were washed twice in cold PBS and homogenized through a micropipette in 50 μl of cold 5% TCA. TCA-precipitable counts were measured in a β-scintillation counter. n represents the total number of 12 somite embryos with open or closed NT morphologies. Most of the SNAP-treated embryos (9/11) show open NT, while in most of the SNAP + FA (5/8) and untreated embryos (4/6) a closed NT is seen. The significance of the difference between the two groups was determined by t-test, $P < 0.001$. (**c**) MS inhibition by NO is concentration dependent and can be alleviated by FA or by vitamin B_{12}. Embryos at the 8–9 somite stage were cultured in pools of five in the presence of labeled 5 M-THF (as described in Figure 1b), treated with the NO donor SNAP at different concentrations (0.5, 2 and 4 mM) or 2 mM SNAP in the presence of 50 μg/ml FA or vitamin B_{12} for 6 h. The results are expressed as mean $\pm$ S.D. calculated from at least four pools of embryos under each treatment. One-way ANOVA with *post hoc* test using Tukey B method revealed significant differences between the treatments: SNAP 2 mM, SNAP 4 mM, FA and B_{12}. (**d**) Dietary FA is required to replenish 5-methyl-tetrahydrofolic acid (5-MTHF) that supplies the methyl group for methionine synthesis. MS is the enzyme that transfers the methyl group from 5-MTHF to homocysteine. Vitamin B_{12} is an essential cofactor of the MS reaction. This reaction seems to be critical for NT closure between the eight and 12 somite stage in the developing chick embryo. Pathological NO levels appear to poison B_{12}, inhibiting the carbon flow by MS reaction and consequently interfering with normal NT closure

interpretation of the results led us to the conclusion that the inhibition of the one carbon flow by NO in cultured embryos may be at the cofactor level of the MS reaction.

The interrelations between NO levels, FA availability and NT closure defect suggested in this letter are illustrated in Figure 1d. Adequate one carbon unit flow, which is mediated by MS and FA dependent, is required for normal NT closure between the eight and 12 somite-embryo stage. Although we cannot precisely specify the NO levels that are required for the normal process of NT closure, it is clear that high NO levels that are capable of poisoning B_{12}, an essential cofactor of the MS enzyme, interfere with the NT closure process. FA is clearly required to prevent the deleterious effect of high NO on NT closure. The observations described here shed light on the molecular mechanisms that underlie FA deficiency and human congenital NT defects, and could give at least one molecular explanation for the necessity of FA dietary supplementation during pregnancy, and how this can prevent these devastating human congenital defects.

Acknowledgements

We thank Tal Beckerman for her technical help. This research was supported by the Israel Science Foundation Grant 6/10-17.2 and by The Adams Super Center for Brain Studies award, Tel Aviv University.

M Weil[*,1], *R Abeles*[1], *A Nachmany*[1], *V Gold*[1] *and E Michael*[1]

[1] Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel

[*] Corresponding author: M Weil. Tel: +972 3 6406981; Fax: +972 3 6422046; E-mail: miguelw@tauex.tau.ac.il

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