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Trophic and proliferative perturbations of in vivo/in vitro cephalic neural crest cells after ethanol exposure are prevented by Neurotrophin 3

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ABSTRACT

Neural crest cells (NCCs), a transient population that migrates from the developing neural tube, distributes through the embryo and differentiates into many derivatives, are clearly involved in the damage induced by prenatal exposure to ethanol. The aim of this work was to evaluate alterations of trophic parameters of in vivo (in ovo) and in vitro NCCs exposed to teratogenic ethanol doses, and their possible prevention by trophic factor treatment.

Chick embryos of 24–30 h of incubation were treated during 10 h with 100 mM ethanol, or 40 ng/ml Neurotrophin 3 (NT3), or 10 ng/ml Ciliary Neurotrophic Factor (CNTF), or ethanol plus NT3 or CNTF, or defined medium; then the topographic distribution of NCC apoptosis was assessed using a whole-mount acridine orange supravital method. Cultures of cephalic NCCs were exposed to the same ethanol or NT3, or CNTF treatments, or ethanol plus one of both trophic factors, or N2 medium. A viability assay was performed using the calcein-ethidium test, apoptosis was evaluated with the TUNEL test, and proliferative capacity after BrdU labeling.

After direct exposure of embryos to 100 mM ethanol for 10 h, a high level of NCC apoptosis was coincident with the abnormal closure of the neural tube. These anomalies were prevented in embryos exposed to ethanol plus NT3 but not with CNTF. In NCC cultures, high cell mortality and a diminution of proliferative activity were observed after 3 h of ethanol treatment. Incubation with ethanol plus NT3 (but not with CNTF) prevented NCC mortality as well as a fall in NCC proliferation.

The consequences of direct exposure to ethanol expand data from our and other laboratories, supporting current opinion on the potential risk of alcohol ingestion (even at low doses and/or during a short time), in any period of pregnancy or lactation. Our in vivo/in vitro model encourages us to examine the pathogenic mechanism(s) of the ethanol-exposed embryo as well as the use of trophic factors for the treatment and/or prevention of anomalies induced by prenatal alcohol.

1. Introduction

Neural crest cells (NCCs) form an embryonic cell population which arises from the dorsal neural tube, migrates long distances through particular pathways in the embryo and gives rise to several derivatives, such as neurons and glia of the peripheral nervous system, pigment cells of the skin, craniofacial components and some endocrine cell types (Le Douarin and Kalcheim, 1999). Moreover, NCCs have major clinical relevance since they are involved in both inherited and acquired developmental human abnormalities (Antony and Hansen, 2000; Dunty et al., 2001; Dunty et al., 2002; Wentzel and Eriksson, 2009), globally known as the Neurocristopathy family (Bolande, 1997). It is also well known that NCCs exhibit a high proliferative rate (Paglini and Rovasio, 1994a,b, 1999) and intrinsic (physiological) apoptotic behavior (Cartwright and Smith, 1995b; Cartwright et al., 1998; Graham et al., 1993; Hirata and Hall, 2000), which makes this cell population potentially labile (Rovasio and Battiato, 2002).

Among the exogenous agents harmful to NCC development, ethanol occupies a key role in teratological studies since prenatal ethanol exposure results in a pattern of anomalies known as Fetal Alcohol Syndrome (FAS) (Jones and Smith, 1973). This embryopathology involves varying degrees of growth retardation, brain and craniofacial malformation, as well as mental health dysfunction, and it is known that many anatomical/functional anomalies of FAS clearly involve cranial NCC-derived tissues (Giles et al., 2008; NIAAA, 2000; Sulik, 2005).

Animal models have played a significant role in determining the phenotypic characteristics and biological consequences of prenatal alcohol exposure. FAS has been reproduced in mammals (Sulik, 2005), frogs (Nakatsuji, 1983) and chickens (Cartwright and Smith, 1995a;

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Rovasio and Battiato, 1995, 1996, 2002). However, there is limited and indirect data or speculative conclusions about the factors involved in the prenatal mechanism of action of ethanol at cellular and molecular levels (Dunty et al., 2001, 2002; Goodlett and Horn, 2001; Kumada et al., 2006, 2007), even though a well-documented effect of ethanol is the inhibition of cell proliferation in the brain (Bonthius and West, 1991; Miller, 1995), cerebellar neurons (Goodlett and Horn, 2001), and neural tube of chick embryos (Giles et al., 2008).

Previous studies from our laboratory have demonstrated that, after direct treatment with ethanol, embryos showed malformations of the neural tube, reduced numbers and impaired distribution of NCCs, as well as significant and permanent structural and dynamic in vitro changes (Rovasio and Battiato, 1995, 1996, 2002). Ethanol exposure is also associated with the increased apoptosis of NCCs (Cartwright and Smith, 1995a,b) and neural tube cells (Giles et al., 2008) in a dose- and time-dependent manner. Experimental data has shown that some cell types can be rescued from apoptosis and other cell perturbations when ethanol is administered together with trophic factors (Heaton et al., 2003, 2004; Kilburn et al., 2006; Luo et al., 1997; McAlhany et al., 2000; McGough et al., 2008). It is also known that Neurotrophin 3 (NT3) null mice are deficient in skin innervation and NCC-derived Merkel cells, displaying significant cell loss associated with immunocytochemical and ultrastructural apoptotic changes (Halata et al., 2005). On the other hand, it has been shown that cutaneous overexpression of NT3 selectively rescues most of the skin sensory innervation in NT3 knockout mice (Krimm et al., 2000). Ciliary neurotrophic factor (CNTF) is also a well-known trophic molecule that promotes survival of neural (Kassen et al., 2009) and non-neural cells (Rezende et al., 2007). Taken together, these data emphasize the importance of a strategy aimed at preventing/rescuing ethanol-induced NCC perturbations by trophic factor treatment.

The purpose of this study was to determine the action of ethanol on the apoptosis pattern in early chick embryo development stages as well as on in vitro NCC viability and proliferative parameters, and the possible role of trophic factor supply. We found that the ethanol-induced increase in apoptosis, as well as the low proliferative capacity of NCCs, may be reverted by simultaneous treatment with NT3.

2. Materials and methods

2.1. Whole embryo culture, determination of apoptosis and morphology

The topographic distribution of in vivo NCC early apoptosis was assessed by acridine orange supravital intake (Cartwright and Smith, 1995b; Graham et al., 1993), on fertile Cobb line chick eggs pre-incubated for 24–30 h (stages 7–8 HH) (Hamburger and Hamilton, 1951) at 38 °C and 80% humidity, and cultured in a modified Auerbach's shell-less culture system (Battiato et al., 1996; Rovasio and Battiato, 1995). Briefly, the whole egg was transferred to a bowl, and a flat ring of 4% agarose[1] in phosphate buffer solution (PBS) (20-mm outer diameter, 10-mm inner diameter; 2 mm thick) was placed over the blastoderm, forming a small container in which 200 µl of treatment solution was poured. Groups of embryos were treated with: (1) 200 µl of N2 basal medium (Bottenstein and Sato, 1979) (50% DMEM and 50% F12 media, plus 15 mM sodium bicarbonate, 15 mM Hepes buffer, 50 IU/ml G sodium penicillin and 50 µg/ml streptomycin sulfate). (2) 200 µl of N2 medium with a final concentration of 100 mM ethanol. (3) 200 µl of N2 medium with a final concentration of 40 ng/ml of NT3, or 10 ng/ml of CNTF. (4) 200 µl of N2 medium with both 100 mM ethanol and 40 ng/ml of NT3, or 10 ng/ml of CNTF (protection experiments). In all groups, re-incubation was performed to complete 40–50 h (stage 11–12 HH) of embryo development. As a control, after the initial egg pre-incubation time, one group was uninterrupted following the in ovo incubation without treatment; no differences were observed between in ovo and shell-less cultured control groups. For an in ovo alternative experimental group, other eggs were injected with 100 µl of PBS or ethanol in N2 medium into the yolk, estimating an equivalent final concentration of ethanol in relation to total egg volume (Rovasio and Battiato, 1995); no significant differences were observed in the parameters studied between shell-less and in ovo ethanol treatments.

At the end of the experimental time, a sample of 100 µl of fluid was taken from the immediate surrounding milieu of the embryo and the concentration of ethanol was verified by a head-space gas chromatography as explained (Pueta et al., 2011). Then, each embryo was excised, washed with warm PBS, and incubated with 1 ml of N2 medium containing 0.5 µg of acridine orange, at 37 °C during 30 min. After washing twice with PBS, each embryo was whole-mounted between slide and coverslip using anti-bleaching medium (Molecular Probes, Eugene, OR), and was observed and recorded using an Olympus BX-50 microscope (Olympus Corp., Shinjuku-ku, Tokyo, Japan) with fluorescence filter for rhodamine (excitatory filter = 510–550 nm and barrier filter = 590 nm).

Normal/abnormal morphology of whole embryo was double-blind evaluated under a high power stereoscopic microscope, using as a normal pattern the external features described and illustrated in the Hamburger and Hamilton series (Hamburger and Hamilton, 1951).

A group of embryos was submitted to immunolabeling of NCCs with NC-1 antibody (Vincent et al., 1983). Briefly, after fixation for 3 h with 4% paraformaldehyde in PBS and prior piercing of the ectoderm with a microneedle, whole mounted chick embryos were rinsed with PBS and incubated in a wet chamber at room temperature with blocking solution (1% bovine serum albumin and 1.5% Glycine in PBS) 3 times, 1 h each. They were then incubated with NC-1 monoclonal antibody-containing ascitic fluid diluted 1/100 with PBS for 12 h, washed in blocking solution 3 times, 1 h each, and incubated with secondary anti-mouse IgGAM antibody conjugate with fluorescein isothiocyanate (FITC) for 12 h at 4 °C. After PBS washing, preparations were mounted with coverslip using anti-bleaching medium and observed with a fluorescence filter for FITC (excitatory filter = 450–480 nm and barrier filter = 515 nm). Recordings were performed using T-MAX or Ektachrome 400 ASA (Kodak, Rochester, NY) films, then digitized with a SnapScan e50 scanner (Agfa Gevaert, NV, Mortsel, Belgium). Alternatively, images were captured using a Hamamatsu C2400 (Hamamatsu Photonics, Hamamatsu City, Japan) video-camera and submitted to image analyses with the SigmaScan-Pro (SPSS, Chicago, IL) software, according to previous descriptions (Rovasio and Battiato, 2002). To insure the homogeneity of results, the embryos of different experimental groups were simultaneously immunolabeled and mounted between glass and coverglass applying the same protocol technique. Briefly, the last step of the whole embryo mounting was performed under a stereomicroscope, placing strips of coverglass of equivalent thickness to that of the embryo as lateral barriers, in order to assure a homogeneous height and to avoid geometrical distortions of the specimen. Then, whole embryos were imaged, maintaining the uniformity of optical parameters, without further computational handling. Some embryos were also submitted to conventional study techniques with transmission electron microscopy.

2.2. Cultures of neural crest cells and treatments

To characterize the trophic and survival parameters of in vitro ethanol-exposed cells during the early migratory stage, primary cultures of cranial NCCs were performed according to descriptions elsewhere (Rovasio and Battiato, 2002; Rovasio et al., 1983). Briefly, fertile chick eggs were incubated as described above to obtain 10 to 13 somite-pair embryos (stages 10–11 HH). Blastoderms and the surrounding membranes were then excised and washed with PBS,

[1] All chemicals were from Sigma Chem. Co. (St Louis, MO), except when another source is stated.