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Cadmium-induced gene expression changes in the mouse embryo, and the influence of pretreatment with zinc

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Abstract

Cadmium (Cd) administered to female C57BL/6 mice on gestation day 8 induces a high incidence of anterior neural tube defects (exencephaly). This adverse effect can be attenuated by maternal pretreatment with zinc (Zn). In this study we used replicated microarray analysis and real-time PCR to investigate gene expression changes induced in the embryo 5 and 10 h after maternal Cd exposure in the absence or presence of Zn pretreatment. We report nine genes with a transcriptional response induced by Cd, none of which was influenced by Zn pretreatment, and two genes induced only by combined maternal Cd exposure and Zn pretreatment. We discuss the results in relation to the possibility that Cd is largely excluded from the embryo, that the teratogenic effects of Cd may be secondary to toxicity in extraembryonic tissues, and that the primary protective role of Zn may not be to reverse Cd-induced transcription in the embryo.

Keywords: Cadmium; Exencephaly; Microarray; Mouse embryo; Neural tube defects; Toxicogenomics; Zinc

1. Introduction

Cadmium (Cd) and valproic acid (VPA) have long been used as model substances for the induction of neural tube defects (NTD) in the mouse embryo [1,2]. Gene expression changes induced in the embryo by the exposure to such agents could shed light on the mechanisms behind NTD, and also serve as biomarkers of teratogenicity. In a recent microarray study we found that the expression of many genes is altered in the mouse embryo after treatment with VPA, and that the proteins encoded by several of these genes appeared relevant to processes involved in neural tube formation and closure [3].

When administered by intraperitoneal (i.p.) injection to mice on day 8 of gestation, which is the stage of highest sensitivity to induction of anterior NTD (exencephaly) by both agents [2,4], VPA accumulates in the embryonic neuroepithelium [5], whereas Cd selectively accumulates in the endodermal epithelium lining the embryonic primitive gut and extraembryonic yolk sac [6]. Even if Cd induces apoptosis in the closing neural tube, signs of this toxic response is not found at the site of Cd accumulation in the anterior visceral endoderm [7]. Although reaching the yolk sac from the maternal blood, injected Cd appears to no longer gain access to the embryo after the vitelline duct closes around day 9.5 [6]. Fetal growth retardation induced by the feeding of Cd to rats throughout gestation has been found not to be attributed to elevated Cd concentrations in the fetus [8], and the direct injection of Cd into the rat fetus in late gestation has been found to induce a lower incidence of fetal death than subcutaneous injection of Cd in the mother [9]. Thus, it has been suggested that the teratogenic effects of Cd may be primarily mediated by toxicity in extraembryonic tissues [6,10,11] and disturbed transfer of zinc (Zn) and other nutrients to the embryo [6,10–14]. Interestingly, also VPA has been associated with disturbed Zn distribution [15].

We previously reported that the incidence of NTD following injection of Cd (4 mg/kg $CdCl_2$ i.p.) on day 8 of gestation in mice was greatly reduced (from 79% to 8%, as evaluated 72 h after Cd exposure) when the mothers were given an injection of Zn (8 mg/kg $ZnCl_2$ i.p.) 2 h before the administration of

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Cd [16]. Embryos with closed neural tubes were about three times more common 24 h after Cd exposure in the presence of Zn pretreatment, even if neural tube closure appeared to be delayed (as indicated by an approximately 10-fold higher number of embryos with open neural tubes) compared with control embryos [16]. The attenuation of the Cd-induced teratogenicity in the presence of Zn was found to be accompanied by less DNA damage and apoptosis in the embryo, and by the reversal of some Cd-induced gene expression changes in the head of the embryo [16].

In the present study we use microarray analysis and real-time PCR to investigate the gene expression changes induced in the mouse embryo by maternal Cd exposure on day 8 of gestation in the absence and presence of Zn pretreatment. Given the differential accumulation of Cd and VPA in the embryo (see above), especially with regard to the neuroepithelium [5,6], we have an interesting opportunity to explore whether both of these NTD-inducing agents regulate similar genes in the embryo during the process leading to NTD, and whether genes known from mouse genetic models of NTD [17] to be critically involved in neural tube formation and closure are involved. We discuss the reported genes also in the light of previously known Cd actions and the observed Zn influence. With the possible exception of the metallothionein-encoding genes *Mt1* and *Mt2*, we report virtually no concordance between the gene expression responses detected in the mouse embryo 5 h (microarrays) or 10 h (real-time PCR) after Cd exposure, and those previously reported [3] to be induced 6 h (microarrays and real-time PCR) after a similar VPA exposure. We therefore address the possibility that Cd exerts its adverse effects in the embryo through largely different modes of action than VPA, despite their common toxic endpoint (exencephaly). With the possible exception of *Ccng1*, the gene encoding the cell cycle regulator cyclin G1, we report no genes for which the Cd-induced expression change in the embryo appears to be simply reversed by Zn pretreatment. We also raise the possibility that Cd exposure in the presence of Zn pretreatment induces embryonic expression of genes not induced by either metal alone.

2. Materials and methods

2.1. Animals

The studies were approved by the Local Ethics Committee for Animal Research, and the experiments complied with current laws of Sweden. C57BL/6 original breeding pairs were purchased from B&K Universal (Solna, Sweden). At our own facilities, the mice were maintained at constant temperature (24–26 °C) and humidity (30–40%) on a 12-h light cycle (11:00 pm–11:00 am), and given food and water ad libitum. Male and female mice were mated from 8:00 am to 10:00 am, after which the female mice were checked for the presence of a vaginal plug, the presence of which was considered as evidence that mating had taken place. The day of the vaginal plug was designated day 0 of gestation, with the 10:00 am time point being equal to 0 days post coitum (d.p.c).

2.2. Treatments

$CdCl_2$ and $ZnCl_2$ obtained from Sigma Chemical Co. (St. Louis, MO, USA) were dissolved at 1 mg/ml in sterile saline (0.9% NaCl). In the morning of day 8 of gestation, the dams were randomized into four groups according to a 2 × 2 factorial design and at 8:00 am (7 days 22 h post coitum) received a first intraperitoneal (i.p.) injection [8.0 ml/kg body weight (b.w.)] of vehicle (v) only or 8.0 mg (59.1 μmol Zn^{2+})/kg b.w. of $ZnCl_2$ (Zn), and 2 h later (8.0 d.p.c.) a second i.p. injection (4.0 ml/kg b.w.) of vehicle (v) only or 4.0 mg (22 μmol Cd^{2+})/kg b.w. of $CdCl_2$ (Cd).

2.3. Isolation of embryos

Dams were killed by cervical dislocation 5 h (for microarrays) or 10 h (for real-time PCR) after the second injection (see above). In the following we refer to these time points as 5 and 10 h after maternal Cd exposure, respectively, regardless if the dam received $CdCl_2$ or vehicle only (saline) at the time (8.0 d.p.c.) of exposure. The uterus was quickly transferred to cold phosphate-buffered saline, pH 7.4, and the embryos dissected out, removing extraembryonic tissues. The embryos were quickly scored according to primitive streak ranking staging [18], and any embryos ranked as 'primitive streak' were excluded. The number of excluded embryos in each treatment group was low. No apparent differences between treatment groups were noted. The isolated embryos were immediately homogenized in TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA), and stored at −80 °C until further processing.

2.4. RNA preparation

Total RNA was isolated according to the manufacturer's instructions (http://www.invitrogen.com/content/sfs/manuals/15596026.pdf). RNA concentrations were determined by spectrophotometer, and RNA quality was checked using the Agilent 2100 Bioanalyzer and the RNA 6000 LabChip® kit (Agilent Technologies, Palo Alto, CA, USA). Only high quality RNA with no signs of degradation, as defined by Agilent's Application Note (https://www.chem.agilent.com/temp/rad471DE/00023567.pdf), was used for further experiments.

2.5. Experimental design

For each of the four treatment groups [vehicle (v + v), Zn (Zn + v), Cd (v + Cd), and Zn + Cd; see above], two biologically independent samples were generated, each created by pooling all embryos from three similarly treated dams (see discussion in ref. [3] for advantages and limitations with this type of pooling strategy), resulting in eight pools (P1–P8). Using a closed loop design [19], four array hybridizations were done for each of the four comparisons, addressing biological and technical variation through a crossed dye reversal strategy adopted from Churchill [20]. This design is a trade-off between the need for biological and technical replication while keeping down animal consumption and microarray use. In addition, the vehicle control and Zn + Cd groups were directly compared through two microarray hybridizations with dye-reversal. This resulted in a total of 18 hybridizations.

2.6. Microarrays

Spotted cDNA microarrays [mouse 15K array (http://www.microarrays.ca/products/types.html#M15K)], containing the National Institute on Aging (NIA) 15K mouse cDNA clone set (http://lgsun.grc.nia.nih.gov/cDNA/15k.html; [21] of ∼15,000 developmentally expressed mouse genes, spotted in duplicate, were purchased from the Microarray Centre, University Health Network, Toronto, Canada; http://www.microarrays.ca/).

2.7. cDNA synthesis and microarray hybridization

Labeling of cDNA for microarray hybridization was done using the 3DNA™ Array 900 Expression Array Detection Kit (Genisphere Inc., Hatfield, PA, USA) according to the manufacturer's protocol and recommendations. Briefly, total RNA (6–8 μg) was oligo-dT-primed with Cy3- or Cy5-capture-sequence primer, and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). For cDNA hybridization, equal amounts of each cDNA (corresponding to 1.4 μg of each total RNA) were mixed with 1 μl mouse Cot-1 DNA (Invitrogen Life Technologies, Carlsbad, CA, USA) and Enhanced hybridization buffer (Genisphere). After hybridization at 60 °C for