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Prenatal cadmium exposure dysregulates sonic hedgehog and Wnt/ β -catenin signaling in the thymus resulting in altered thymocyte development¹

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Abstract

Cadmium (Cd) is both an environmental pollutant as well as a component of cigarette smoke. Although evidence demonstrates that adult exposure to Cd causes changes in the immune system, there are limited reports in the literature of immunomodulatory effects of prenatal exposure to Cd. The sonic hedgehog (Shh) and Wnt/ β -catenin pathways are required for thymocyte maturation. Several studies have demonstrated that Cd exposure affects these pathways in different organ systems. This study was designed to investigate the effect of prenatal Cd exposure on thymocyte development, and to determine if these effects were linked to dysregulation of Shh and Wnt/ β -catenin pathways. Pregnant C57Bl/6 mice were exposed to an environmentally relevant dose (10 ppm) of Cd throughout pregnancy and effects on the thymus were assessed on the day of birth. Thymocyte phenotype was determined by flow cytometry. A Gli: luciferase reporter cell line was used to measure Shh signaling. Transcription of target genes and translation of key components of both signaling pathways was assessed using real-time RT-PCR and western blot, respectively. Prenatal Cd exposure increased the number of CD4⁺ cells and a subpopulation of double-negative cells (DN; CD4⁺CD8⁻), DN4 (CD44⁺CD25⁻). Shh and Wnt/ β -catenin signaling were both decreased in the thymus. Target genes of Shh (*Patched1* and *Gli1*) and Wnt/ β -catenin (*c-fos*, and *c-myc*) were affected differentially among thymocyte subpopulations. These findings suggest that prenatal exposure to Cd dysregulates two signaling pathways in the thymus, resulting in altered thymocyte development.

Keywords

cadmium; prenatal; thymocyte development; sonic hedgehog; Wnt

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CONFLICT OF INTEREST STATEMENT

The authors have no financial conflict of interest.

INTRODUCTION

Cadmium (Cd), a heavy metal, is an environmental pollutant due to its widespread and continual use. It currently ranks seventh on the Agency of Toxic Substances and Disease Registry/Environmental Protection Agency (ATSDR/EPA) list of Hazardous Substances (ATSDR, 2007). Humans normally absorb Cd into the body either by ingestion or inhalation (Lauwerys *et al.*, 1986). The daily intake is estimated to be approximately 10-50 µg, but can reach levels of 200-1000 µg in highly contaminated areas (Nordberg, 2006). Cd concentrations in food normally range from 10-20 µg, while a cigarette contains 1-2 µg. Cd levels in soils, particularly areas in which phosphate fertilizers have been applied, can range from 10 to 200 µg/g (Cook, 1995). Cd has a half-life of 15-20 years in humans, which contributes to its toxicity (Jin *et al.*, 1998). Cd exposure during gestation leads to a variety of fetal malformations in rodents, such as postaxial forelimb ectrodactyly (Scott *et al.*, 2005), delayed effects on renal function (Jacquillet *et al.*, 2007), and sensorimotor development (Minetti and Reale, 2006). Despite the known teratogenic effects of Cd, only one report concerning the effect of Cd exposure during gestation on the immune system was found (Soukupova *et al.*, 1991). In that study, pregnant ICR mice were administered Cd on day 16 of gestation, and the immune responses of their offspring were tested at 4 and 8 weeks of age. Proliferative responses of spleen cells to mitogens and activity of peritoneal macrophages were increased, while delayed type hypersensitivity to sheep red blood cells after immunization was decreased, in Cd-exposed offspring.

The thymus is a target organ of Cd-induced toxicity. Cd-treatment of adult rats results in damage to the thymus as well as changes in the proliferation rate of thymocytes (Morselt *et al.*, 1988). T-cell development in the thymus is essential for the establishment and maintenance of the adaptive immune system. Thymocytes mature through a series of stages defined by expression of cell surface markers CD4 and CD8. The most immature thymocytes are CD4⁻CD8⁻ double-negative (DN). This population gives rise to CD4⁺CD8⁺ double-positive (DP) cells, which then give rise to mature CD4⁺CD8⁻ single-positive (SP) and CD4⁻CD8⁺ SP cells. Thymocytes can pass through an immature single positive (ISP) stage following the DP stage; however, this population decreases to a negligible level prior to birth and remains insignificant throughout adulthood (Xiao *et al.*, 2003). The DN population is further subdivided in mice based on the expression of surface markers CD25 and CD44: CD44⁺CD25⁻ (DN1) cells differentiate into CD44⁺CD25⁺ (DN2) cells, which then develop into CD44⁻CD25⁺ (DN3) cells, which differentiate into CD44⁻CD25⁻ (DN4) cells. Several *in vivo* experiments in adult rodents have demonstrated that Cd causes decreased thymus weight as well as thymic atrophy (Borgman *et al.*, 1986; Mackova *et al.*, 1996; Liu *et al.*, 1999). In adult mice, Dong *et al.* (2001) observed a decrease in DP cells. Pathak and Khandelwal (2008) supported these findings and demonstrated that Cd exposure increased the number of DN cells.

The Hedgehog (Hh) and Wnt family proteins act as morphogens during thymocyte development. The Hh family of secreted intercellular signaling molecules is an important regulator in patterning and organogenesis during animal development. There are three mammalian Hh proteins: sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh). Hh proteins share a common signaling pathway. They bind to their surface receptor Patched (Ptc), in order to signal to neighboring cells (Marigo *et al.*, 1996; Stone *et al.*, 1996). Ptc releases its suppression of the cell surface molecule Smoothed (Smo), enabling the Hh signal to be transmitted into the target cell (van den Heuvel and Ingham, 1996). This transduction is regulated by complex interactions and modifications of many cytoplasmic proteins ultimately resulting in the activation of members of the Gli family of zinc finger transcription factors (Gli 1-3) (Ingham and McMahon, 2001). When Hh protein is absent, Ptc inhibits the ability of Smo to signal (Chen and Struhl, 1998; Taipale *et al.*, 2002). Shh signaling,

in particular, is critical in the development of thymocytes and T-cell activation (Shah *et al.*, 2004). Shh proteins act as regulators at several stages of T-cell development in the thymus.

In addition to Hh proteins, the Wnt family of glycoproteins is involved in regulating thymocyte maturation (Staal *et al.*, 2002). Wnt proteins are secreted morphogens that are involved in a variety of cell activities in development. Wnt signals are transduced through at least three different signaling pathways; however, the canonical β -catenin/T-cell factor-lymphoid enhancer factor (TCF-LEF) primarily functions during thymocyte development. The canonical pathway is stimulated by Wnt proteins that bind to cell surface Frizzled (Fz) receptors (Bhanot *et al.*, 1996). Signaling through Fz receptors following Wnt binding results in the stabilization of β -catenin (Behrens *et al.*, 1996). β -catenin is a multifunctional protein that can enter the nucleus and function as a transcription factor (Novak and Dedhar, 1999). When Wnt signaling is absent, free β -catenin is phosphorylated by glycogen synthase kinase-3 β (GSK-3 β) and quickly targeted for proteosomal degradation (Ikeda *et al.*, 1998; Amit *et al.*, 2002; Gao *et al.*, 2002). Inhibition of the Wnt pathway results in reduced DN proliferation and differentiation, and decreased DP survival (Verbeek *et al.*, 1995; Okamura *et al.*, 1998; Schilham *et al.*, 1998).

Cd has been found to downregulate Shh signaling in mouse embryonic limb buds (Scott *et al.*, 2005) and zebrafish embryos (Yu *et al.*, 2006), as well as to dysregulate the Wnt pathway in chick embryonic periderm (Thompson *et al.*, 2008) and proximal tubule cells (Prozialeck *et al.*, 2003; Thevenod *et al.*, 2007). The purpose of this study was to investigate the effect of prenatal Cd exposure on thymocyte development, and to determine if these effects were linked to dysregulation of the Shh and Wnt/ β -catenin pathways.

MATERIALS AND METHODS

Breeding and Dosing Methodology

C57Bl/6 mice at 8-10 weeks of age were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). The C57Bl/6 strain of mouse was used for these experiments due to its reported teratogenic susceptibility to Cd treatment (Hovland *et al.*, 1999). Mice were allowed to acclimate on site for at least one week. Two females were placed in a cage with one male for 72 hours to maximize pregnancy rate. Females were inspected for a vaginal plug and if present, this day was declared as gestational day 0. For each experiment, ten dams were used as controls, having free access to deionized distilled water (ddH₂O), while ten additional dams had free access to 10 ppm of Cd as CdCl₂ (Sigma-Aldrich; St. Louis, MO) dissolved in ddH₂O. The dose of 10 ppm was chosen because it is the greatest concentration that will elicit immunomodulatory effects in adult rodents without causing systemic effects (Lafuente *et al.*, 2003). In addition, 10 ppm is a relatively low and environmentally realistic concentration (Thijssen *et al.*, 2007). Cd administration was stopped at birth. At post-natal day 0 (PND0), which was <12 h following birth, 3 offspring from each litter were euthanized and thymi were removed.

Cd concentration in kidneys

Cd levels in the kidneys of dams, and the kidneys and livers of offspring were measured following parturition, due to Cd's known accumulation and retention in these organs (Webb, 1972). The purpose of determining how much Cd was retained in the kidneys of the dams was to verify that the dams were consuming approximately equal amounts of water, and thus, Cd dosing was consistent between dams. To measure Cd content, kidney and liver samples were dissolved in 2 ml of 70% nitric acid. The acidified samples were neutralized in 5 ml of ddH₂O and filtered through Whatman no.1 paper. Samples were then diluted to volume in 10 ml ddH₂O. Cd concentrations were measured using an inductively coupled plasma optical

emission spectrometry (ICP-OES) (model P400 Perkin Elmer, Shelton, CT). The minimum level of detection of the ICP-OES for Cd is 2.5 ppb.

Sex determination

The sex of newborn mice was determined by amplifying the Y-chromosome-specific SRY gene by PCR. The end piece of each tail (~5 mm) was trimmed and lysed in DirectPCR Lysis Reagent (Viagen Biotech, Inc., Los Angeles, CA) containing freshly prepared 0.2-0.4 mg/ml Proteinase K (Sigma-Aldrich, St. Louis, MO). After incubation at 55°C for 5-6 h with vigorous shaking, crude lysates were incubated at 85°C for 45 m by floating the tubes on a water bath. One µl of DNA was used in a 25 µl PCR reaction also containing 0.5 µM SRY primers (F: 5'-GAGAGCATGGAGGGCCAT-3'; R: 5'-CCACTCCTCTGTGACACT-3'), 0.5 µM β-actin primers (F: 5'-TGTGATGGTGGGAATGGGTCAG-3'; R: 5'-TTTGATGTCACGATTTC-3'), 15 µl of 5 PRIME HotMasterMix (Gaithersburg, MD) and molecular biology grade water. β-actin, a housekeeping gene, was used as a positive control for the PCR reaction. The PCR reaction was performed with a GenAmp PCR System 9700 thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA). PCR program was 1.5 m at 94°C, then 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 m at 72°C, then 5 m at 72°C. Ten µl of the PCR product underwent electrophoresis on a 1% agarose gel and was visualized under UV illumination using ethidium bromide staining (5µg/ml).

Tissue Isolation and Cell Preparation

Thymi were harvested from euthanized mice and single cell suspensions prepared. The organs from each mouse were kept separate. Red blood cells were lysed using an ammonium chloride lysis buffer. Viable cells were enumerated using trypan blue and a hemacytometer.

Whole thymic lysates and cytoplasmic/nuclear extraction

Thymic lysates used in the Shh-Light 2 cell assay and western blots were prepared by homogenization and sonication of whole thymi in 200 µl serum-free Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manassas, VA) supplemented with 2 µl Protease Inhibitor Cocktail (Sigma-Aldrich). Protein levels were quantitated using the BCA Protein Assay Kit (Pierce, Rockford, IL). Cytoplasmic/nuclear extracts of thymocytes were prepared from single-cell suspensions. Cells were harvested and washed in 1x PBS. The pellet was resuspended in 500 µl of cytoplasm extraction buffer (10mM HEPES, 40 mM KCl, 2 mM MgCl₂, 10% glycerol, 1 mM NaPPi, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM NaVO₄, 1 mM NaF, 1 mM PMSF, ddH₂O to 50 ml), then vortexed vigorously. Samples were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant (cytoplasmic fraction) was removed. The remaining pellet was washed several times in 1x PBS. The pellet was resuspended in 500 µl of nuclear extraction buffer (10 mM HEPES, 500 mM NaCl, 1% Triton-X 100, 10% glycerol, 1 mM NaPPi, 1 µg/ml, pepstatin, 1 µg/ml, aprotinin, 1 µg/ml leupeptin, 1 mM NaVO₄, 1 mM NaF, 1 mM PMSF, ddH₂O to 50 ml), then sonicated for 30 s with a 1 min rest 8 times. The pellet was centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant (nuclear fraction) was removed. Protein levels of extracts were quantified using the 2-D Quant Kit (GE Healthcare, Piscataway, NJ).

Cell staining, flow cytometry, and cell sorting

Single cell suspensions of thymocytes were prepared as described above. Thymocytes were stained using combinations of the following fluorochrome directly conjugated antibodies: anti-CD45-biotin (eBioscience; San Diego, CA), anti-streptavidin-Pacific Blue (Invitrogen; Carlsbad, CA), anti-CD44-PE-Cy5 (eBioscience), anti-CD25-PE-Cy7 (eBioscience), anti-CD4-FITC (BD Biosciences Pharmingen; San Jose, CA), and anti-CD8-APC (BD Biosciences Pharmingen). Anti-CD45 was used to identify leukocytes then SP and DP cell subpopulations

were identified using anti-CD4 and anti-CD8. To identify the different DN subpopulations, anti-CD44 and anti-CD25 were determined on the CD4⁺CD8⁻ population. Cells (1×10^6) were stained using the following procedure: cells were washed with PBSAz (phosphate buffered saline containing 2% FBS and 0.2% sodium azide) and then incubated with whole rat and mouse IgG (Jackson ImmunoResearch, West Grove, PA) for 30 minutes on ice to block Fc receptors, followed by a PBSAz wash. The cells were incubated for 30 minutes on ice with fluorochrome labeled antibodies. The cells were washed several times with PBSAz and fixed overnight at 4°C with 0.4% paraformaldehyde. The paraformaldehyde was removed and cells resuspended in PBSAz. Stained cells were analyzed using a FACSaria and FACSDiva software (BD Biosciences Pharmingen). A total of 10,000 events were collected for each sample.

For experiments represented in Table 1, Figures 1, 2D and E, and 3D and E, purified populations of thymocytes (DN, DP, CD4⁺, and CD8⁺) were sorted by flow cytometry using the antibodies listed above. Table 1 shows the purity of the sorted populations of a typical flow cytometry sorting run. Cells were stained, washed, and resuspended in FACS buffer (PBS, 1% FBS, 25 mM HEPES, 2.5 mM EDTA). Final purity of all cell populations was in excess of 92%.

Shh signaling

Shh signaling was assessed using Shh-Light 2 cells (ATCC), an NIH-3T3-derived stable cell line containing integrated Gli-luciferase and constitutive *Renilla* luciferase reporters. Shh-Light 2 cells (1×10^4 cells/well) were cultured in DMEM containing 10% FBS to maximal density (full contact inhibition of growth) in a 96-well plate. The media was then removed and replaced by thymic cell lysate containing 10 µg of protein in 100 µl of DMEM containing 0.5% FBS and 5 mM HEPES buffer (pH 7.4) added to the wells in duplicate. A duplicate set of wells contained only 100 µl DMEM containing 0.5% FBS and 5 mM HEPES served as background. Another set of duplicate wells contained 10 µg protein from a control offspring to which was added 5µM cyclopamine-KAAD (Calbiochem, San Diego, CA), an inhibitor of Shh signaling, to verify that luciferase was based on Shh signaling. Cells were incubated for 36 h. Luciferase activity was measured and normalized to a *Renilla* control using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and Synergy HT Multi-Detection Microplate Reader (Biotek, Winooski, VT)

Western blots

A 20-30 µg aliquot of each whole thymic lysate or cytoplasmic/nuclear extract was boiled for 5 min to denature the proteins and electrophoresed through a 12% Tris polyacrylamide gel with a 5% stacking gel at 25 mAmps for 18 h. Proteins were transferred onto Hybond-P membranes (Amersham Pharmacia, Piscataway, NJ) at 0.5 amps for 3 h. A semi-random order of the samples was used to be sure that differences were not due to gel variations. Blots were washed in TBS for 5 min at room temperature, blocked for 1 h in TBS + 0.1% Tween 20 (TBS/T) plus 5% dry milk at room temperature and then washed three times in TBS/T. Blots were incubated overnight at 4°C with primary antibodies specific for anti-Shh-N-terminal (R & D Systems, Minneapolis, MN), anti-Gli1 (Novus Biologicals, Littleton, CO), anti-active-β-catenin, clone 8E7 that detects dephosphorylated β-catenin on Ser37 and Thr41 (Millipore, Temecula, CA), anti-phospho-β-catenin that detects pSer³⁷ (Sigma), anti-GAPDH (Santa Cruz, Santa Cruz, CA) or anti-Oct-1 (Santa Cruz) in TBS/T plus 5% BSA or milk. The next day, blots were washed three times in TBS/T, incubated for 1 h at room temperature with anti-Biotin (Cell Signaling Technology, Inc., Danvers, MA) and either a goat anti-mouse IgG-horseradish peroxidase (HRP) (Millipore) or an anti-rabbit IgG-HRP antibody (Sigma). Finally, the blots were washed three times in TBS/T and developed using Phototope-HRP detection kit for western blots (Cell Signaling Technology, Inc) and bands were visualized on X-Ray film (BioMax MR, Eastman Kodak Company). Densitometric analysis was performed using ImageJ software (NIH,

Bethesda, MD). Whole thymic and cytoplasmic protein levels were normalized to GAPDH protein levels, while nuclear extracts were normalized to Oct-1 protein levels for each sample.

Real-time RT-PCR

RNA isolation from whole thymi and thymocytes was performed using an RNeasy Mini Kit (Qiagen, Valencia, CA). Total cDNA was synthesized from 40 ng RNA/sample with Sensiscript Reverse Transcriptase (Qiagen) according to the instructions of the commercial supplier, and used as a target in PCR amplifications. QuantiTect Primer Assays for Ptc1, Gli1, Wnt10b, c-jun, c-fos, c-myc, and β -actin were purchased from Qiagen. All PCR reactions were performed on a Lightcycler 2.0 Real-time PCR System (Roche, Indianapolis, IN) using QuantiTect SYBR Green Master Mix (Qiagen) under the following conditions: 15 min at 95°C to activate HotStarTaq DNA polymerase then 45 cycles of 15 s at 94°C, 20 s at 55°C, and 20 s at 72°C. Melting curve analysis of PCR products was performed to verify their specificity and identity. β -actin was used as the reference gene.

Statistics

Results are expressed as mean $\pm$ S.E.M. For Figures 1A-C, 2A-C, and 3A and B, a mean of the data from 3 mice/litter was taken and used as the N=1 data for the corresponding dam. For Figures 2D, E and 3C-E, a mean of the data from 2 mice/litter was taken and used as the N=1 data for the corresponding dam. Statistical analyses comparing the values for a particular cell population, protein expression, or luciferase activity between Cd-exposed (single dose) and control offspring were performed using the t-test. An alpha value of $p \leq 0.05$ was considered significant. Relative gene expression data was analyzed using the $2^{-\Delta\Delta C_T}$ method described in Livak and Schmittgen (Livak and Schmittgen, 2001). The fold-change reported includes the standard error. The luciferase assay and real-time RT-PCR was repeated 2 times, while all other experiments were repeated at least three times.

RESULTS

Tissue Cd levels

The average Cd concentration for the dams was 4.37 ± 0.76 (SEM) $\mu\text{g/g}$ kidney tissues, verifying that Cd dosing was consistent between dams. Analysis of Cd levels in the offspring was attempted; however, no Cd was detected in the kidneys and liver Cd levels were slightly above the minimum level of detection for the ICP-OES (2.5 ppb) when livers from 3 offspring from Cd-treated dams were pooled. Cd was not detected in the offspring from control dams. This finding demonstrates that the offspring were exposed to Cd; however, transplacental transfer was very low.

Effect of prenatal Cd exposure on thymocyte phenotype in post-natal day 0 offspring

Table 1 reflects a representative flow cytometry sorting run. As evident by post-sort purity percentages, this technique resulted in highly purified subpopulations of each of the thymocyte populations under study.

Thymocyte phenotype of representative offspring from each litter was measured by cell surface expression of CD4 and CD8 using flow cytometry. Total thymocyte number was not significantly different between Cd-treated and control offspring ($7.74 \pm 0.53 \times 10^6$ vs. $8.75 \pm 0.63 \times 10^6$, respectively). The number of CD4⁺ cells was significantly increased in Cd-treated offspring compared to control offspring [$3.09 \pm 0.44 \times 10^6$ vs $1.48 \pm 0.21 \times 10^6$ ($p < 0.05$), respectively] (Figure 1A). The number of CD8⁺ cells showed a trend ($p = 0.06$) for decrease in Cd-treated offspring. The significantly increased number of CD4⁺ cells and the decreased number of CD8⁺ cells resulted in a nearly 10-fold increase in the CD4⁺/CD8⁺ ratio [Cd-treated,

93.14±22.94 vs. control, 9.53±3.63 ($p<0.001$)] (Figure 1B). Analysis of the DN subpopulations based on CD44 and CD25 cell surface expression using flow cytometry showed that Cd-treated offspring had significantly more DN4 cells ($3.64\pm0.12 \times 10^5$) compared to control offspring ($1.93\pm0.08 \times 10^5$) ($p<0.01$) (Figure 1C). The DN3 population also showed a trend ($p=0.06$) for increase Cd-treated offspring. It should be noted that none of these effects were sex specific.

Effect of prenatal Cd exposure on Shh signaling in the thymus

Shh signaling activity was measured in whole thymic lysates using Shh-Light 2 cells, which produce luciferase in response to active Shh proteins. In the thymus, Shh production is restricted to thymic epithelial cells (TECs), thus this assay quantified Shh signaling level in TECs. Thymic lysates from Cd-treated offspring had an approximate 24% decrease in Shh signaling ability compared to control offspring (Figure 2A). Cyclopamine (5 μ M), an inhibitor of Shh signaling, eliminated total luciferase activity in control thymic lysates (not shown), indicating that the observed luciferase activity was based on Shh signaling.

Effect of prenatal Cd exposure on Shh and Gli1 protein expression in the thymus

To determine if the decrease in Shh signaling activity was due to a decrease in Shh protein levels, thymic lysates were analyzed by western blot. In Shh producing cells, full length Shh is autocatalytically cleaved to generate an active N-terminal fragment (Shh-N) modified by cholesterol. Using an antibody specific for Shh-N, western blot analysis demonstrated comparable levels of Shh protein in Cd-treated offspring compared to controls (Figure 2B). It should be noted that the variability observed in Cd-treated offspring was not correlated to sex, litter, or Cd concentration in the dam.

When Shh is present, Gli1 proteins are translocated to the nucleus where they activate target gene transcription. When Shh is absent, Gli1 proteins are sequestered in the cytoplasm by a multiprotein complex that contains suppressor of fused (SUFU). To determine if decreased Shh signaling from TECs affected Gli1 expression, protein level analysis in whole thymic lysates was performed by western blot. Gli1 protein levels were not significantly different between Cd-treated and control offspring (Figure 2C). To determine whether nuclear translocation of Gli1 was affected, total thymocytes were fractionated and Gli1 protein levels were determined in cytoplasm and nuclear fractions. The protein levels in each fraction were comparable between Cd-treated and control offspring (data not shown).

Effect of prenatal Cd exposure on Shh target genes

mRNA levels of the Shh target genes *Gli1* and *Ptc1* were measured in sorted thymocyte subpopulations using real-time RT-PCR. Analysis of *Gli1* mRNA levels showed nearly a 5-fold increase in relative expression in DN thymocytes of Cd-treated offspring (9.37 ± 0.91) compared to control offspring (1.0 ± 0.75), and a greater than 3-fold increase in relative expression in CD8⁺ thymocytes of Cd-treated offspring (7.67 ± 0.89) compared to control offspring (1.0 ± 0.97) (Figure 2D). Similarly, analysis of *Ptc1* mRNA showed nearly a 5-fold increase in DN thymocytes of Cd-treated offspring (6.5 ± 1.01) compared to control offspring (1.0 ± 0.16), and a more than 6-fold increase in CD8⁺ thymocytes of Cd-treated offspring (15.24 ± 1.44) compared to control offspring (1.0 ± 1.23) (Figure 2E). In summary, prenatal Cd exposure increased expression of Shh target genes, *Gli1* and *Ptc1*, in DN and CD8⁺ thymocytes, despite decreased Shh signaling in TECs.

Effect of prenatal Cd exposure on β -catenin protein expression in thymocytes

In order to determine the effect of prenatal Cd exposure on the Wnt/ β -catenin pathway, active β -catenin (ABC) and phosphorylated β -catenin (phospho- β -catenin) protein levels were determined in the nucleus and cytoplasm, respectively. Western blot analysis showed a

significant decrease in ABC levels in thymocyte nuclei of Cd-treated offspring compared to control offspring [2.04 ± 0.25 vs. 3.71 ± 0.21 ($p < 0.05$), respectively] (Figure 3A). It should be noted that analysis of ABC levels in the cytoplasm and the total lysate showed no significant difference between Cd-treated and control offspring (data not shown). In addition, western blot analysis of phospho- β -catenin showed a significant increase in protein levels in thymocyte cytoplasm of Cd-treated offspring compared to control offspring [0.48 ± 0.07 vs. 0.19 ± 0.07 ($p < 0.05$), respectively] (Figure 3B). In summary, prenatal Cd exposure increased phosphorylation of β -catenin in the cytoplasm, resulting in decreased ABC levels in the nucleus.

Effect of prenatal Cd exposure on Wnt10b expression in the thymus

To determine whether the alteration in β -catenin levels in Cd-treated offspring was due to a decrease in Wnt levels, transcriptional levels of Wnt10b were determined by real-time RT-PCR. Wnt10b was chosen for analysis due to its expression by mouse TECs at embryonic day 15 (Pongracz *et al.*, 2003) and its involvement in the Wnt/ β -catenin pathway (Austin *et al.*, 1997; Van Den Berg *et al.*, 1998). Analysis of Wnt10b mRNA expression showed a greater than 4-fold decrease in thymi of Cd-treated offspring compared to thymi of control offspring (0.02 ± 0.08 vs. 1.0 ± 0.57 , respectively) (Figure 3C). When isolated thymocytes were analyzed for Wnt10b mRNA transcripts, no transcript was detected by real-time RT-PCR, thus the Wnt10b that was detected in whole thymic lysates probably came from the TECs present in the whole thymi.

Effect of prenatal Cd exposure on Wnt/ β -catenin target genes

Wnt/ β -catenin target genes *c-fos*, *c-jun*, and *c-myc* were measured in sorted thymocyte subpopulations using real-time RT-PCR. Analysis of *c-fos* showed a 3-fold increase in relative expression in DN and DP thymocytes of Cd-treated offspring (DN, 4.0 ± 0.58 ; DP, 4.9 ± 0.68) compared to control offspring (DN, 1.0 ± 0.12 ; DP, 1.0 ± 0.35), as well as a greater than 5-fold increase in CD4⁺ thymocytes of Cd-treated offspring (12.1 ± 1.18) compared to control offspring (1.0 ± 0.97) (Figure 3D). Analysis of *c-myc* showed a 3-fold increase in relative expression in DN and DP thymocytes of Cd-treated offspring (DN, 4.4 ± 0.41 ; DP, 4.4 ± 0.44) compared to control offspring (DN, 1 ± 0.34 ; DP, 1 ± 0.3) (Figure 3E). Analysis of *c-jun* showed no significant difference between Cd-treated and control offspring (data not shown). In summary, prenatal Cd exposure increased at least one Wnt/ β -catenin target gene in all thymocyte subpopulations analyzed except CD8⁺ thymocytes.

DISCUSSION

Immunotoxic effects following Cd exposure in adult animals are well documented; however, there have been no studies that investigate the immunomodulatory effects of gestational Cd exposure to the offspring on thymocyte development. Studies demonstrating a dysregulation of Shh (Scott *et al.*, 2005) and Wnt/ β -catenin (Thompson *et al.*, 2008) signaling by prenatal Cd exposure, coupled with the requirement for Shh (Outram *et al.*, 2000) and Wnt/ β -catenin (Oosterwegel *et al.*, 1991; Verbeek *et al.*, 1995; Hattori *et al.*, 1996; Ioannidis *et al.*, 2001) in thymocyte development, led to the hypothesis that prenatal Cd exposure dysregulates these signaling pathways in the offspring, leading to changes in thymocyte phenotype. To our knowledge, this is the first study to link prenatal Cd exposure to changes in thymocyte development and to dysregulation of the Wnt/ β -catenin pathway in a mouse model.

Our ex vivo analysis of thymocyte phenotype showed that prenatal Cd exposure increased the number of CD4⁺ and DN4⁺ cells, as well as the CD4⁺/CD8⁺ ratio. Other studies that have examined the effect of direct in vitro Cd exposure on thymocyte phenotype, however, report a decrease in DP cells (Dong *et al.*, 2001), an increase in DN cells (Pathak and Khandelwal,

2008), and a decrease in the CD4⁺/CD8⁺ ratio (Dong *et al.*, 2001; Pathak and Khandelwal, 2008). In those studies, primary thymocytes from 3-6 week old male Balb/c mice were exposed in vitro to various Cd concentrations (5-50 μ M) for several time intervals (3-24 h). However, the relevance of these studies to the interpretation of those reported in this study is questionable because of the differences in experimental design such as in vitro versus in vivo exposure, Cd dose, length of exposure, mouse strain, and developmental stage of the exposed cells. In addition, in vitro exposure would not account for possible effects on the thymic epithelium in addition to the thymocytes that as described below, could have a significant impact on the phenotype. In our study, thymocytes are being exposed to an indirect, environmentally relevant concentration of Cd (Nordberg, 2006). Although Cd was detected in pooled livers of the offspring, the level was close to the minimum level of detection, thus it is likely that any possible direct Cd effect is minimal to negligible. The increased CD4⁺/CD8⁺ ratio observed in our study could have several consequences in cell-mediated immunity and T-cell host response to infection including an increased chance of developing autoimmune disease and allergies, as well as an increased susceptibility to viruses and tumor cells. Analysis of prenatal Cd exposure on immune cell development and function at later developmental stages (PND14 and 49) has demonstrated that prenatal exposure to environmentally relevant Cd levels causes persistent immunomodulatory effects in murine offspring (unpublished data).

Analysis of Shh signaling showed that prenatal Cd exposure dysregulates several components of this pathway (Figure 4A). Shh is produced by thymic epithelial cells (TECs), while its receptor molecules Ptc and Smo are expressed by thymocytes (Outram *et al.*, 2000), thus the decreased Shh signaling activity observed in the Shh-Light 2 cell assay can be attributed to a down-regulation in signal transduction from the Shh-producing cells to the Shh-receiving cells (Figure 4A, top). The downregulation of Shh target gene *Wnt10b* (Figure 4B) in the whole thymus further supports an inhibitory role of prenatal Cd exposure on Shh signaling from the epithelium. Several processing events must occur for proper secretion of the processed Shh (Shh-Np). Such events include: post-translation cleavage of the original 45 kDa protein along with the sequential addition of cholesterol (Porter *et al.*, 1995) and palmitoylate (Pepinsky *et al.*, 1998), and the further requirement of the protein Dispatched for secretion (Burke *et al.*, 1999), and multimerization of the Shh protein (Zeng *et al.*, 2001). Analysis of Shh protein in thymic lysates indicates that the active cholesterol-modified form of Shh protein levels are unchanged. However, any of the other steps following the cholesterol modification may be altered following prenatal Cd exposure.

Cells near the source of Shh secretion can modulate the range of the signal by upregulating their expression of Ptc, which can sequester Shh and thereby prevent it from spreading further (Chen and Struhl, 1996). Studies determining the expression pattern of *Smo* and the *Gli* genes suggest that the thymocytes responding to the Shh signal are DN cells and CD8⁺ cells (Outram *et al.*, 2000). In addition, analysis of Shh^{-/-} thymi showed that Shh is necessary for efficient proliferation of DN thymocytes (Shah *et al.*, 2004). In our study, analysis of Shh target genes *Ptc1* and *Gli1* in Cd-treated offspring showed a significant upregulation of both genes in the DN and CD8⁺ cell population, while there was no difference in either gene in the DP and CD4⁺ cell populations (Figure 4A, bottom). Thus, we hypothesized that in an environment where Shh signaling is decreased, DN and CD8⁺ cells may upregulate *Ptc1* in order to sequester the limited signal. Upregulation of *Gli1* transcription indicates that Shh signaling is increased in the DN and CD8⁺ cell populations, despite the decreased Shh signal from the TECs and the unchanged protein level of Gli1 in the total thymocyte population.

Analysis of Wnt/ β -catenin signaling showed that prenatal Cd exposure dysregulates several components of this pathway (Figure 4B). Wnt proteins begin as precursors containing an N-terminal hydrophobic signal peptide that directs the immature protein to the endoplasmic reticulum (ER) and several post-translational modifications are required to convert them into

hydrophobic proteins, which is essential for their biological activity (Tanaka *et al.*, 2000; Willert *et al.*, 2003; Zhai *et al.*, 2004). Transport and secretion of the Wnt protein in secretory vesicles is controlled by the multi-pass transmembrane protein Wntless (Wls)/Evendless interrupted (Evi) (Ching and Nusse, 2006). In our study, we observed reduced mRNA levels of Wnt10b in TECs (Figure 4B, top), along with decreased Wnt/ β -catenin signaling in thymocytes as determined by increased cytoplasmic phospho- β -catenin and decreased nuclear ABC (Figure 4B, bottom). A decrease in active Wnt ligand would lead to degradation of β -catenin in the cytoplasm, which is observed in prenatal Cd-treated offspring. In addition, others have demonstrated that ABC enhances the generation of CD8⁺ cells from DP cells (Mulroy *et al.*, 2003), thus decreased ABC may be responsible for the increased CD4⁺/CD8⁺ ratio we observed in prenatal Cd-treated offspring.

Cd has been shown to be mitogenic and to influence the expression of genes, especially the cellular proto-oncogenes, also known as the immediate early response genes, that encode nuclear transcription factors and influence subsequent expression of other genes (Vogt and Bos, 1989). Cd-induced accumulation of transcripts of *c-fos*, *c-jun*, and *c-myc* has been reported in several cell types of animals and humans (Jin and Ringertz, 1990; Tang and Enger, 1993; Matsuoka and Call, 1995; Wang and Templeton, 1998; Achanzar *et al.*, 2000; Joseph *et al.*, 2001). These genes are generally associated with cell proliferation, thus their induction indicates a mechanism by which Cd may promote the development of cancer. In our study, *c-fos* was upregulated in DN, DP, and CD4⁺ cells, but was unaffected in CD8⁺ cells; *c-myc* was upregulated in DN and DP cells, but was unaffected in CD4⁺ and CD8⁺ cells; and *c-jun* was unaffected in all cell populations (Figure 4B, bottom). In summation, a significant increase in at least one of these immediate early response genes was present in every thymocyte population analyzed except for the CD8⁺ population. Interestingly, thymocyte phenotype analysis showed that this was the only population that had a trend ($p=0.06$) for decrease following prenatal Cd exposure. Although *c-fos* and *c-myc* are known Wnt/ β -catenin target genes, it is unlikely that their upregulation is due to an increase in ABC levels since prenatal Cd exposure caused a decrease in ABC and an increase in phospho- β -catenin protein levels in total thymocytes when analyzed via western blot. β -catenin, together with DNA-binding T cell factor/lymphoid enhancer factor (TCF/LEF) family proteins, functions as a transcription factor to control Wnt target genes, thus, prenatal Cd exposure may be upregulating TCF/LEF or downregulating repressors such as Groucho/TLE which would result in upregulation of the proto-oncogenes. It has also been demonstrated that overexpression of cellular proto-oncogenes by Cd is mediated by the elevation of intracellular levels of superoxide anion, hydrogen peroxide, and calcium (Joseph *et al.*, 2001).

In summary, we exposed pregnant mice to an environmentally relevant dose of Cd throughout pregnancy and analyzed the effects on thymocyte phenotype of the offspring. We also examined the effect of prenatal Cd exposure on two signaling pathways necessary for proper thymocyte maturation. We demonstrated that prenatal Cd exposure alters Shh and Wnt/ β -catenin signaling which we hypothesize is the cause of the aberrant thymocyte development. The overall upregulation of immediate early response genes suggests that prenatal Cd also affects an intracellular component of the thymocyte independent of the effects on the thymic epithelium. Although it is known that Shh and Wnt/ β -catenin signaling influences fetal thymocyte development, the specific time points at which they play a role and the regulation of their components, as well as their interaction with each other need further investigation. Due to Hh and Wnt/ β -catenin signaling being highly conserved among organ systems, it is plausible that prenatal Cd exposure disrupts these pathways in other organs, resulting in developmental malformations, increased cell proliferation, and possibly cancer.

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Figure 1.

Thymocyte phenotype of PND0 offspring. Thymocytes were isolated from newborn mice (< 12 h old) whose mothers were exposed to 10 ppm Cd throughout pregnancy. Single cell suspensions were prepared for flow cytometry analysis. (A) Thymocyte phenotype was determined based on CD4 and CD8 cell surface expression. *, $p < 0.05$ (B) CD4⁺/CD8⁺ ratio was based on thymocyte number. *, $p < 0.001$ (C) DN subpopulation phenotype was determined based on CD44 and CD25 cell surface expression. *, $p < 0.01$

Figure 2.

Shh signaling activity in PND0 offspring. (A-C) Thymic lysates were prepared from newborn mice (< 12 h old) whose mothers were exposed to 10 ppm Cd throughout pregnancy. (A) Shh signaling was measured by luciferase activity in Shh-Light 2 cells. Each bar represents the mean $\pm$ SEM. The mean value of control samples was given a value of 100. Data is representative of 2 independent experiments where N=7 in each group. *, $p < 0.05$ (B) Shh protein levels, and (C) Gli1 protein levels were determined by western blot. GAPDH was used as loading control. Each bar represents the mean $\pm$ SEM. Data is representative of 3 independent experiments where N=3 in each group. The ratio of N-Shh protein to GAPDH protein (B) and the ratio of Gli1 protein to GAPDH protein (C) was determined using densitometry of the

detected bands. (D and E) Hh target gene expression in thymocyte subpopulations. Primers specific for (D) Gli1 and (E) Ptc1 were used to determine their expression in DN, DP, CD4⁺, and CD8⁺ thymocyte populations. β -actin was used as the reference gene. Relative quantification of target genes was calculated using $2^{-\Delta\Delta C_T}$. Data is representative of 2 independent experiments where N=2 in each group. *, ≥ 3 -fold difference between control and Cd-treated groups.

Figure 3.

Wnt/β-catenin signaling in PND0 offspring. (A and B) Active β-catenin and phospho-β-catenin expression in thymocytes of PND0 offspring. Thymocytes were isolated from newborn mice (< 12 h old) whose mothers were exposed to 10 ppm Cd throughout pregnancy. Cytoplasmic and nuclear fractions were extracted and analyzed by western blot. GAPDH (cytoplasmic) and Oct-1 (nuclear) were used as loading controls. Each bar represents the mean ± SEM. Data is representative of 3 independent experiments where N=3 in each group. The ratio of active β-catenin protein to Oct-1 protein in nuclear fractions (A) and the ratio of phospho-β-catenin protein to GAPDH protein in cytoplasmic fractions (B) was determined using densitometry of the detected bands. *, $p < 0.05$ (C and D) Wnt10b expression in thymus of PND0 offspring. (C) Thymic lysates were prepared from newborn mice (< 12 h old). Protein levels were determined by western blot. GAPDH was used as loading control. Each bar represents the mean ± SEM. Data is representative of 3 independent experiments where N=3 in each group. (D) Primers specific for Wnt10b were used to determine mRNA expression in whole thymus. β-actin was used as the reference gene. Relative quantification of target genes was calculated using $2^{-\Delta\Delta C_T}$. Data is representative of 2 independent experiments where N=2 in each group.

*, ≥ 3 -fold difference between control and Cd-treated groups. (E and F) Wnt/ β -catenin target gene expression in thymocyte subpopulations of PND0 offspring. Primers specific for (E) c-fos and (F) c-myc were used to determine their expression in DN, DP, CD4⁺, and CD8⁺ thymocyte populations. β -actin was used as the reference gene. Relative quantification of target genes was calculated using $2^{-\Delta\Delta C_T}$. Data is representative of 2 independent experiments where N=2 in each group. *, ≥ 3 -fold difference between control and Cd-treated groups.

Figure 4.

Proposed model for the effect of prenatal Cd exposure on Shh and Wnt/β-catenin signaling in the thymus. (A) Shh signaling in the thymic epithelium and thymocytes, (B) Wnt/β-catenin signaling in the thymic epithelium and thymocytes. [], no change; ↑/↓, increase/decrease in protein or gene expression; —, inhibition of signaling; genes are italicized.

Table 1

Typical purity of thymocyte analysis to determine the phenotype of PND0 offspring

Thymocyte population	Pre-sort profiles	Post-sort purities
DN	8.0%	92.0%
DP	84.4%	97.1%
CD4*	5.0%	92.9%
CD8*	2.6%	93.3%

Thymocytes were isolated from newborn mice (< 12 h old) whose mothers were exposed to 10 ppm Cd throughout pregnancy. Single cell suspensions were prepared for flow cytometry analysis.