Prenatal cadmium exposure dysregulates sonic hedgehog and Wnt/beta-catenin signaling in the thymus resulting in immunomodulatory effects

Miranda Leah Hanson
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Prenatal cadmium exposure dysregulates sonic hedgehog and Wnt/beta-catenin signaling in the thymus resulting in immunomodulatory effects

Miranda Leah Hanson

Dissertation submitted to the School of Medicine at West Virginia University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Immunology and Microbial Pathogenesis

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ABSTRACT

Prenatal cadmium exposure dysregulates sonic hedgehog and Wnt/β-catenin signaling in the thymus resulting in immunomodulatory effects

Miranda Leah Hanson

Cadmium (Cd) is both an environmental pollutant as well as a component of cigarette smoke. Recent data suggests increased cancer risks and increased mortality in environmentally exposed populations. 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. Our experiments were 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. In addition, longer term effects of prenatal Cd on the immune system were investigated. Pregnant C57Bl/6 mice were exposed to an environmentally relevant dose (10 ppm) of Cd during pregnancy and effects on the thymus of the offspring were assessed on post-natal day 0 (PND0), while effects on the thymus and spleen were assessed on PND14 and 49. On PND0, 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. On PND14 and 49, thymocyte and splenocyte phenotypes were analyzed, and cytokine production of splenic T cells was determined by ELISA. On PND0, prenatal Cd exposure increased the number of CD4+ cells and a subpopulation of double-negative cells (DN; CD4^-CD8^-). Shh and Wnt/β-catenin signaling were both decreased in the thymus; however, this was not due to altered Shh and Wnt protein levels. Target genes of Shh and Wnt/β-catenin were affected differentially among thymocyte subpopulations. On PND14 and 49, prenatal Cd exposure increased the number of DN thymocytes. In the spleen, prenatal Cd exposure had a cell type-, sex-specific effect on splenocyte phenotype and cytokine production. Collectively, these findings suggest that even very low exposure to Cd during gestation dysregulates two signaling pathways in the thymus resulting in altered thymocyte development, and this dysregulation can result in long term detrimental effects on the immune system of the offspring.
LIST OF ABBREVIATIONS

APC  Adenomatous polyposis coli
AXIN  Axis inhibitor
BMP  bone morphogenic protein
BOC  bioregional CDON binding protein
BRG  Brahma-related gene
β-TRCP  beta-transducin repeat containing protein
C  carboxyl-terminal
Ca  calcium
CBP  CREB-binding protein
CD  cluster of differentiation
CK  Casein kinase
Cl2  chloride
CMJ  cortico-medullary junction
CREB  cyclic-AMP-responsive-element binding protein
cTEC  cortical thymic epithelial cell
Cu  Copper
DC  dendritic cell
Dhh  Desert hedgehog
Disp  Dispatched
DKK1  Dikkopf-1
DMT1  divalent metal transport 1
DN  double-negative
DP  double-positive
DVL  Dishevelled
E  embryonic day
E2  Estradiol
ER  estrogen receptor, or endoplasmic reticulum
ERK  extracellular signal regulated kinase
Evi  Evenness interrupted
Fe  Iron
Fgf  fibroblast growth factor
Frizzled  Fz or FZD
FTOC  fetal thymic organ culture
GAS1  growth arrest specific1
GSH  Glutathione
GSK3β  glycogen synthase kinase 3 beta
h  Hour
HSC  hematopoietic stem cells
ICAT  Inhibitor of β-catenin
IFN-γ  interferon-gamma
Ihh  Indian hedgehog
IL interleukin
ISP immature single positive
kDa kilodalton
LD lethal dose
LEF lymphoid enhancer factor
LGS Legless
LRP low density lipoprotein receptor-related protein
MHC major histocompatibility complex
min minute
MT metallothionein
mTEC medullary thymic epithelial cell
N amino-terminal
NC neural crest
NK natural killer
NO nitric oxide
PGE$_2$ prostaglandin E2
ppb parts per billion
ppm parts per million
Ptc/PTCH Patched
Pygo Pygopus
RAG Recombination-activating gene
ROS reactive oxygen species
SC subcutaneous
SCID severe combined immunodeficient
SCZ subcapsular zone
Shh Sonic hedgehog
Ski Skinny hedgehog
Smo Smoothened
SO$_4$ sulfate
SP single-positive
SUFU Suppressor of fused
TCF T-cell factor
TCR T cell receptor
TEC thymic epithelial cell
TGFβ Transforming growth factor beta
TLE Transducin-like Enhancer of Split
TNF-α tumor necrosis factor-alpha
Wg Wingless
Wls Wntless
wt1 Wilms tumor suppressor
Zn zinc
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CHAPTER 1: Literature Review I
Development of the Immune System

Thymus

The immune system originates from bone-marrow-resident hematopoietic stem cells (HSCs) that give rise to progenitor populations with increasingly restricted lineage potential, ultimately leading to production of all lineages of mature blood cells. The majority of hematopoietic lineages mature in the marrow, while the thymus is the primary lymphoid organ for T cell generation (Miller and Osoba, 1967). The thymus is the site of marrow-derived progenitors and is responsible for maintaining multistage lineage commitment and differentiation steps to produce mature, self-tolerant, functional T cells. Even though the thymus is a vital part of the hematopoietic system, the thymus does not contain self-renewing stem cells. In adults, the thymus is constantly being seeded from the bone marrow by small numbers of progenitor cells, which reach the thymus via the blood stream (Wallis et al., 1975) and enter the cortex-medulla boundary (Ceredig and Schreyer, 1984; Lind et al., 2001). The thymus is divided into an outer cortex, where most differentiation takes place, and an inner medulla, where newly formed cells undergo final maturation before exiting and seeding peripheral lymphoid organs (Scollay and Godfrey, 1995). The pattern of movement of developing thymocytes differs between the adult and fetal thymus, with progenitor cells entering the fetal thymus through the outside of the thymus rudiment (Suniara et al., 1999; Manley, 2000), and in the adult thymus through blood vessels in the medulla or perimedullary cortex (Lind et al., 2001; Petrie, 2003; Benz et al., 2004).

During murine embryogenesis, the thymus primordium is formed between embryonic day (E) 10.5 and E11.5 from the third pharyngeal pouch endoderm, which is
a process that depends on interactions with the surrounding neural crest (NC)-derived mesenchyme. NC cells migrate from the dorsal portion of the neural tube during embryogenesis and contribute to a variety of neuronal and non-neuronal cell types. NC-derived mesenchyme is necessary for thymus organogenesis and thymus function (Manley, 2000; Owen et al., 2000; Anderson and Jenkinson, 2001; Petrie, 2002). The thymic epithelium is derived from the embryonic endodermal layer (Blackburn and Manley, 2004). Differentiation is dependent on and controlled by cellular interactions (Bockman, 1997). The roof of the endodermal yolk sac is folded into the expanding embryo as the foregut. The thymus rudiment begins to express Foxn1 at about E11.5. Foxn1 encodes a transcription factor whose function is essential for subsequent epithelial differentiation; without it, colonization of the rudiment by thymocyte progenitors fails (Bleul and Boehm, 2000), and thymopoiesis is aborted, resulting in severe immunodeficiency (Nehls et al., 1996). It is still unknown which signal(s) determine(s) the site and size of the thymic rudiment and whether these or other signals stimulate Foxn1 expression. Genetic abnormalities involving the formation of pharyngeal pouches, such as deficiencies in the transcriptional regulators, Tbx1, Pbx1, Eya1, and Six1, also have an impact on thymus formation (Manley et al., 2004; Arnold et al., 2006; Zou et al., 2006). Wnt and bone morphogenic protein (BMP) signals have also been implicated in initiating (Balciunaite et al., 2002) and/or maintaining (Bleul and Boehm, 2005; Patel et al., 2006) Foxn1 expression in the thymic epithelium. During embryonic development, Sonic hedgehog (Shh) signaling is involved in restricting the size of the thymic field as measured by Foxn1 expression (Moore-Scott and Manley, 2005).
At E12, the thymic rudiment is first colonized by lymphocyte progenitors (Jotereau et al., 1987), which enter through the capsule via a chemoattractive mechanism (Liu et al., 2006). At this point, the rudiment does not have histologically defined cortex and medulla regions that are present in the adult thymus. Rather, it contains mostly thymic epithelial cell (TEC) progenitors that subsequently undergo an ill-defined lineage commitment and differentiation program, resulting in distinct cortical and medullary TEC subsets, which are the major cells of the stroma (Rossi et al., 2006). The epithelial cells attract lymphoid precursor cells that are transported to this region through the bloodstream. By E13.5 in mice, the parathyroid and thymus are separated into physically distinct organs. Shortly following this, the thymus reaches its approximate adult position within the embryo. Other nonhematopoietic stromal elements, such as fibroblast and endothelial cells, and distinct hematopoietic-derived myeloid dendritic cells (DCs) and macrophages also contribute to the final thymic structure. Macrophages are distributed throughout the cortex and medulla where they are involved in phagocytosis of apoptotic cells (Surh and Sprent, 1994). DCs are predominately located in medullary regions, but they reach into cortico-medullary areas and have access to immature thymocytes (Kyewski et al., 1987; Shortman and Vremec, 1991; Surh and Sprent, 1994).

Thymus growth in the mouse takes place in two well defined periods: from E14 to E17-18, and from day 3 to day 6 after birth, as well as another less characteristic period from day 8 to day 14, when the thymus reaches its maximum, steady state size (Penit and Vasseur, 1989). The overall size of the thymus is probably determined by the action of p63 and fibroblast growth factor (Fgf) signaling through the Fgfr2IIIb receptor,
with p63 acting upstream of Fgfr2 (Candi et al., 2007; Senoo et al., 2007). The thymic epithelium is necessary for T cell development which is demonstrated by the phenotype of nude mice, in which disruption of the transcription factor FoxN1 arrests TEC development at an immature progenitor stage and leads to loss of intrathymic T cell development and severe immunodeficiency (Nehls et al., 1996; Su et al., 2003; Bleul et al., 2006). The specialized TEC subsets of the cortex and medulla facilitate distinct phases of T cell development. Thymocytes at different maturation stages occupy distinct regions in the adult thymus, implying that differentiation is coupled with the coordinated migration between microenvironments. Progenitors enter at the corticomedullary junction (CMJ), migrate through the cortex to the outer subcapsular zone (SCZ) during the early progenitor stages, and then move back toward the CMJ and into the medulla (Lind et al., 2001; Petrie and Zuniga-Pflucker, 2007). Cortical TECs (cTECs) support early T cell progenitor commitment and differentiation and play a primary role in β-selection and positive selection processes (Anderson et al., 1993; Anderson et al., 1994). Medullary TECs (mTECs), along with DCs, are needed for effective negative selection during the late stages of development (Derbinski et al., 2001; Gallegos and Bevan, 2004). There are multiple checkpoints during thymocyte maturation to limit the generation of cells having nonfunctional or autoreactive T cell receptor (TCR) complexes. αβ T cell differentiation is characterized by CD4 and CD8 coreceptor expression (Figure 1). Lymphoid progenitor cells that seed the thymus are the immature CD4<sup>−</sup>CD8<sup>−</sup> double-negative (DN) precursor subset (Schmitt and Zuniga-Pflucker, 2005). The first checkpoint during thymocyte development is TCR β-selection which makes sure that only DN thymocytes that have generated a productively
rearranged TCR\(\beta\) chain are selected for further differentiation to the CD4\(^+\)CD8\(^+\) double-positive (DP) stage (Dudley et al., 1994; von Boehmer et al., 1999).

The second checkpoint takes place when DP cells go through positive and negative selection, allowing differentiation of MHC-restricted self-tolerant mature CD4 and CD8 single positive (SP) T cells (Starr et al., 2003). In a mature thymus, ~5% are DN cells, ~80% are DP cells, ~10% are CD4\(^+\) cells, and ~5% are CD8\(^+\) cells (Ceredig et al., 1983). In the mouse, the DN population can be further subdivided into four sequential developmental subsets as follows: DN1 (CD117\(^+\)CD44\(^+\)CD25\(^{-}\)), DN2 (CD117\(^+\)CD44\(^+\)CD25\(^{-}\)), DN3 (CD117\(^{lo}\)CD44\(^{-}\)CD25\(^{+}\)), and DN4 (CD117\(^{-}\)CD44\(^{-}\)CD25\(^{-}\)) (Godfrey et al., 1993). In the human thymus, CD34\(^+\)CD1a\(^{-}\) cells correspond to murine DN1 and DN2 thymocytes and CD34\(^+\)CD1a\(^{+}\) cells are homologous to DN3 thymocytes (Dik et al., 2005). The DN1 cell population has multi-lineage potential, including B-cell, T-cell, myeloid cell, natural killer (NK)-cell and DC potential (Ardavin et al., 1993; Matsuzaki et al., 1993; Moore and Zlotnik, 1995; Shortman and Wu, 1996). The DN2 population lacks B-cell potential but still has NK-cell and DC potential in addition to T cell potential (Wu et al., 1996; Schmitt et al., 2004). Final T-cell lineage commitment occurs at the DN3 stage when extensive rearrangement of TCR\(\beta\), TCR\(\gamma\), and TCR\(\delta\) loci occurs (Ismaili et al., 1996; Capone et al., 1998). DN3 cells with productive TCR\(\beta\) rearrangements give rise to \(\alpha\beta\)-lineage DP
cells that are short lived and the dominant T-lineage cell type in the adult mouse thymus (Huesmann et al., 1991). DN precursors give rise to two distinct T cell lineages, \(\alpha\beta\) and \(\gamma\delta\), however, the exact timing of when this occurs has not been determined. The TCR signal strength determines \(\alpha\beta\) or \(\gamma\delta\) lineage outcome (Haks et al., 2005; Hayes et al., 2005). Relatively weaker signals are associated with the pre-TCR, which is thought to signal autonomously, while stronger signals are associated with the \(\gamma\delta\)-TCR, possibly via ligand engagement (Irving et al., 1998; Yamasaki et al., 2006).

Recombination-activating gene (RAG) 1- and RAG2-mediated rearrangements of the TCR\(\beta\), TCR\(\gamma\), and TCR\(\delta\) loci, which are needed for TCR assembly, are first detected in DN2 cells and continue predominantly during the mostly noncycling DN3 stage (Godfrey et al., 1994; Capone et al., 1998; Livak et al., 1999). Immature thymocytes expressing a functional TCR\(\beta\) chain, which associates with the invariant pre-TCR\(\alpha\) chain and CD3 signaling molecules to form the pre-TCR complex, are selected for \(\alpha\beta\) lineage differentiation (Wu et al., 1996). The pre-TCR mediates the TCR \(\beta\)-selection event by signaling rescue from apoptosis, intensive cellular expansion, termination of TCR\(\beta\) locus recombination, and differentiation to the DP stage (Dudley et al., 1994). In addition to the pre-TCR, signals from chemokines, interleukins, adhesion molecules, and Notch-receptor-ligand-interactions are necessary for differentiation from the DN3 stage to the DP stage (Misslitz et al., 2004; Radtke et al., 2004; Misslitz et al., 2006). In mice with gene deficiencies in Rag1, Rag2, or any component of the pre-TCR complex, severe arrest in \(\alpha\beta\) T cell development at the DN3 stage occurs, thus demonstrating the critical role of pre-TCR formation (von Boehmer et al., 1999; Michie and Zuniga-Pflucker, 2002). During the pre-TCR-induced transition to the DP stage, selected cells
downregulate CD25 and progress through a brief, intermediate immature CD8 single positive (ISP) stage.

Previous studies have established that proliferation is mostly associated with the DN and early DP populations (Scollay and Godfrey, 1995), therefore most cortical DP thymocytes are small nondividing cells. These cells have a finite lifespan of 3-4 days, but are able to undergo positive selection and differentiation into CD4⁺ or CD8⁺ cells (Lundberg and Shortman, 1994; Swat et al., 1994). Continued TCRα chain gene rearrangement has been demonstrated in the DP population, increasing chances of positive selection (Petrie et al., 1993). In regards to proliferation of the DN population, studies have shown that cells that are mostly nondividing following entry into the thymus undergo two phases of proliferation separated by a stable period at the DN3 stage (Penit et al., 1995).

Interactions between thymocytes and stromal cells are essential for the development of both T cell precursors and the thymic stroma. A study in which severe combined immunodeficient (SCID) mice displayed an absence of mTECs until the introduction of normal bone marrow, at which point regeneration of mTECs occurred, demonstrated that development of the thymic medulla is dependent upon the presence of thymocytes (Shores et al., 1991). In addition, absence of medullary areas has been noted in TCRα-deficient mice (Palmer et al., 1993). The importance of the thymocyte-stromal interaction is also evident in positive and negative selection. Two-photon imaging studies of thymocyte behavior showed longer dwell times of thymocytes in a positively selecting environment, indicating that, similar to mature T cells, thymocytes require sustained interactions with their environment for the delivery of selection signals.
(Bousso and Robey, 2004). In general, most negative selection occurs in the medulla once the thymocyte has been positively selected and chosen to commit to the CD4 or CD8 lineage. Negative selection depends on tissue-specific antigen expression by the transcription factor Aire as well as mTEC integrity (Anderson et al., 2002; Kajiura et al., 2004; Akiyama et al., 2005; Kinoshita et al., 2006). The chemokine CCR7 has been shown to be necessary for the migration of the developing thymocyte from the cortex to the medulla (Ueno et al., 2004), and this migration is needed for the establishment of self-tolerance (Kurobe et al., 2006).

Many studies have implicated transcription factors, cytokines, growth factor receptors, signaling molecules, and extracellular matrix molecules as playing important roles in thymocyte maturation, including Wnt molecules, Hh molecules, and FGFs (Staal and Clevers, 2003; Rowbotham et al., 2007b). Also, roles for BMPs and the TGF-β superfamily have been implicated in thymocyte development, mostly for their ability to influence FGF and Wnt-stimulated signals (Tsai et al., 2003; Licona-Limon and Soldevila, 2007).

During the neonatal and early postnatal stages of development, the immune response varies greatly from that of a fully developed adult. One of the major differences is that neonatal T cells have poor cytokine production in comparison to the adult (Bryson et al., 1981) particularly in relation to Th1 cytokines. The underlying mechanisms that account for this deficiency are not fully understood, but appear to derive partly from the secretory functions of the placenta (Wegmann et al., 1993). The relatively poor capacity of neonatal T cells to produce cytokines is considered to contribute to the impaired responses of other neonatal cell populations that depend on
these factors for their functions, e.g. poor IFN-γ production could help to reduce cellular cytotoxicity by NK cells (Yabuhara et al., 1990), and reduced IL-4 has a role in reduced IgE production by neonatal B cells (Pastorelli et al., 1990).

**Spleen**

Splenic development in the mouse begins at E11.5, and at E12.5, it is detectable by the condensation and proliferation of Hox11+ mesenchymal cells as a ridge attached to the dorsal area of the stomach (Roberts et al., 1994). The first hematopoietic cells to populate the mouse spleen appear at around E15.5. Spleen development depends on Hox11+ and in mice lacking this homeobox gene, development of the splenic rudiment begins normally but development arrests between E12.5-13.5, prior to colonization by hematopoietic cells (Roberts et al., 1994). Critical roles in spleen development have also been identified for the transcription factors Wilms tumor suppressor (wt1), Bapx1, and capsulin. Mice lacking the wt1 gene die during late gestation (Herzer et al., 1999). Analysis of wt1-deficient embryos demonstrated that the spleen rudiment formed at E11-12 but involuted by E15 (Herzer et al., 1999). Bapx1 deficiency also results in a variety of developmental defects and is associated with perinatal lethality. Analysis at E11.5 showed that the spleen rudiment failed to form and Hox11 expression could not be detected, suggesting a very early role for Bapx1 (Tribioli and Lufkin, 1999; Lettice et al., 2001). Capsulin-deficient mice also die perinatally (Lu et al., 2000). Capsulin expression was detected within the undifferentiated mesoderm corresponding to the embryonic origin of the spleen in the dorso-lateral wall of the stomach (Lu et al., 2000).
Morphogen Signaling

Thymocyte development and T-cell lineage commitment are influenced by the mammalian homologues of many protein families that were first identified as developmental regulators of *Drosophila*, including Notch (Radtke *et al.*, 1999; Radtke *et al.*, 2004; Guidos, 2006), Wnt (Verbeek *et al.*, 1995; Schilham *et al.*, 1998; Mulroy *et al.*, 2002), BMP (Graf *et al.*, 2002; Hager-Theodorides *et al.*, 2002; Tsai *et al.*, 2003; Cejalvo *et al.*, 2007), and Hh family of proteins (Outram *et al.*, 2000; Sacedon *et al.*, 2003; Shah *et al.*, 2004). The Hh and Wnt family proteins, and the BMPs, act as morphogens during vertebrate embryogenesis and organogenesis by regulating patterning and cell fate. Morphogens are secreted signaling molecules produced at a localized source that specify cell fate in a concentration-dependent manner.

**Hh signaling**

The Hh family of secreted proteins is comprised of intercellular signaling molecules that specify cell fate and patterning during the development of many tissues. In mammals, there are three Hh family members, Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) (Ingham and McMahon, 2001), of which Shh is the best studied. Shh−/− animals have a multitude of defects including cyclopia, poor neural patterning, lack of limb growth, and abnormal organ and foregut development (Ingham and McMahon, 2001). In addition, a variety of diseases and malformations can occur when other genes of the Shh signaling pathway are mutated (Wynshaw-Boris, 2006) (Table 1). In general, the processing and signaling of Hh family
members is similar across the various mammalian paralogs and is also evolutionary conserved. Hh is initially synthesized as a preprotein, which subsequently enters the secretory pathway where the signal sequence is removed to yield an approximately 45 kDa precursor protein (Ingham and McMahon, 2001; Torroja et al., 2005) (Figure 2). The precursor protein undergoes an intramolecular cleavage reaction to generate a 19 kDa amino-terminal polypeptide (Hh-N) and a carboxyl-terminal polypeptide (Hh-C) (Lee et al., 1994; Bumcrot et al., 1995). During this cleavage, cholesterol is covalently attached to the C-terminus of the Hh-Np (p for processed) (Porter et al., 1995). This cleavage takes place in an undefined intracellular compartment. Hh is the only known secreted ligand that is covalently modified by cholesterol. It is likely that this unique

<table>
<thead>
<tr>
<th>Gene mutated</th>
<th>Disease</th>
<th>Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHH</td>
<td>Holoprosencephaly</td>
<td>Variable midline defects (single maxillary incisor, hypotelorism, holoprosencephaly, cyclopia)</td>
</tr>
<tr>
<td>PTCH-1</td>
<td>Gorlin syndrome</td>
<td>Dysmorphic syndrome (short metacarpals, rib defects, broad face, dental abnormalities), cancer predisposition (rhabdomyosarcoma, medulloblastoma)</td>
</tr>
<tr>
<td>PTCH-1 or -2</td>
<td>Cancer</td>
<td>Basal cell carcinomas, medulloblastomas</td>
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<tr>
<td>SMO</td>
<td>Cancer</td>
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<tr>
<td>GLI1</td>
<td>Cancer</td>
<td>Glioblastoma, osteosarcoma, rhabdomyosarcoma, B cell lymphomas</td>
</tr>
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<td>GLI3</td>
<td>Greig syndrome</td>
<td>Hypertelorism, syndactyly, preaxial polydactyly</td>
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<td></td>
<td>Pallister-Hall syndrome</td>
<td>Postaxial polydactyly, syndactyly, hypothalamic hamartomas</td>
</tr>
</tbody>
</table>

Adapted from Wynshaw-Boris (2006)
modification results in many of the unusual biological mechanisms that have been described in the Hh pathway. A palmitoyl acid is also added to the N-terminus of Shh-N (Pepinsky et al., 1998). This palmitoylation is catalyzed by the membrane bound O-acyltransferase commonly known as Skinny Hh (Ski) (Mann and Beachy, 2004; Torroja et al., 2005). Animals lacking Ski function exhibit phenotypes resembling Hh^−/− animals, demonstrating the importance of palmitoylation for transduction of the Hh signal (Pepinsky et al., 1998; Chamoun et al., 2001; Chen et al., 2004). The majority of Hh found in vivo appears to be modified by both cholesterol and palmitate (Pepinsky et al., 1998; Chamoun et al., 2001). The lipid modifications serve an important role in regulating Hh localization and activity. The dually lipidated protein is then secreted from the Shh-producing cell by the 12-pass membrane protein Dispatched (Disp) (Burke et al., 1999). Hh processing occurs normally in Disp^−/− cells, but it is unable to efficiently leave the cell (Ingham and McMahon, 2001; Torroja et al., 2005). Complete loss of Disp function leads to strong Hh loss of function phenotypes, indicating a positive role for Disp in Hh signaling (Torroja et al., 2005; Guerrero and Chiang, 2007). The dually modified Hh is secreted as a multimeric form (Zeng et al.,
2001; Chen et al., 2004; Gallet et al., 2006). Formation of multimeric Hh allows the normally hydrophobic Hh-Np to transmit a signal far from its site of synthesis, as disruption of these multimeric Hh structures results in loss of long-range Hh signaling (Chen et al., 2004; Gallet et al., 2006).

Shh and Ihh are both expressed in the thymus. Shh is produced by the thymic epithelium, and Ihh expression is mainly associated with blood vessels in the medulla (Outram et al., 2000), and more recently has been shown to be produced by DP cells (Outram et al., 2008). Each of the Hh proteins has different expression patterns and essential nonredundant roles during embryogenesis, but do share a common signaling pathway (Figure 3). They bind to their surface receptor Patched (Ptc), in order to signal to neighboring cells (Marigo et al., 1996; Stone, 1996). There are two Patched isoforms, Ptc1 and Ptc2. The mouse and zebrafish homologues of Ptc2 have been reported to be expressed in a partly overlapping pattern with Ptc1 during embryonic development and to be induced by Shh (Concordet et al., 1996; Motoyama et al., 1998). Tissue distribution analysis indicates that Ptc2 is preferentially expressed in the skin and in the testis, where it is likely to mediate the action of Dhh, which is required for germ cell development (Carpenter, 1998). Ptc2 is expressed in the thymus, but its expression is restricted to CD34+ thymic progenitor cells and cortical and medullary cytokeratin-positive epithelial cells (Sacedon et al., 2003). Ptc2 is generally weaker than Ptc1 in its tumor-suppressor ability (Carpenter, 1998). When Hh is present, Ptc (both isoforms) releases its suppression of the cell surface molecule Smoothened (Smo), enabling the Hh signal to be transmitted into the target cell (van den Heuvel and Ingham, 1996) (Figure 3). 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). Gli1 is an activator of transcription whereas Gli2 and Gli3 probably function both as positive and negative regulators of transcription (Koebernick and Pieler, 2002). Gli1 and Gli2 transcription is positively regulated by Hh signaling, while Gli3 transcription is downregulated by Hh (Ruiz i Altaba, 1999). Gli1 is not required to initiate the Hh signal and is itself a target gene of the Hh pathway, so measurement of its transcription can be used as a read-out of Hh signaling in a population of cells. When Hh signaling is absent, Gli2 and Gli3 undergo modification by phosphorylation and cleavage of their transactivation domain so that they function as repressors of target gene transcription (Matise and Joyner, 1999; Koebernick and Pieler, 2002). Further regulation of Gli protein activity is mediated by sequestration of Gli proteins in the cytoplasm by a multiprotein complex that contains suppressor of fused (SUFU), which acts as a negative regulator of the Hh pathway, by Figure 3. Hh signaling pathway
preventing nuclear accumulation of Gli1 and Gli2 (Barnfield et al., 2005). Gli2 and Gli3 are each essential for mouse development and have unique, as well as partially overlapping functions (Mo et al., 1997). Although Gli2 and Gli3 are bifunctional, Gli2 has been reported to act in vivo as a transcriptional activator, while Gli3 mainly functions as a transcriptional repressor (Wang et al., 2000; Bai et al., 2002).

Several factors that influence the response of a cell to the signal received include Hh concentration and length of exposure to the signal (Stamataki et al., 2005). The presence of cell-surface molecules such as CDO (cell-adhesion-molecule-related/downregulated by oncogenes; also called CDON), BOC (bioregional CDON binding protein) and GAS1 (growth-arrest-specific 1) enhances Shh signaling (Tenzen et al., 2006; Allen et al., 2007). In addition, cells can migrate in and out of range of the Hh signal. Cells near the source of Hh secretion can modulate the range of the signal by upregulating their expression of Ptc1, which can sequester Hh and thereby prevent it from spreading further (Chen and Struhl, 1996).

Components of the Hh signaling pathway, including Shh, Ihh, Ptc, Smo, Gli1, Gli2, and Gli3 are expressed in the murine thymus in both the adult and the fetus (Outram et al., 2000; Li et al., 2002a; Li et al., 2002b). Immunohistochemical studies have located Shh production to epithelial cells scattered in the subcapsular region and in the medulla and cortico-medullary junction of both mice and human thymi (Outram et al., 2000; Sacedon et al., 2003; Shah et al., 2004; Andaloussi et al., 2006; Virts et al., 2006). Cell-surface expression of the signal transduction molecule Smo is mostly restricted to DN thymocytes and is downregulated on the subsequent DP and CD4⁺ and CD8⁺ populations. Analysis of DN populations showed that the DN1 cells that are not
committed to the T-cell lineage (Haks et al., 1999) do not express significant levels of Smo (Outram et al., 2000). In DN2 cells, Smo expression is upregulated, but then decreases in the DN3 and DN4 populations, and is then almost undetectable in DP populations (Outram et al., 2000; Andaloussi et al., 2006). Smo expression is then upregulated on SP, with expression on 9% of CD4\(^+\) cells and 20% of CD8\(^+\) cells (Outram et al., 2000). This expression pattern suggests that the thymocytes that are responding to the Hh signal are the DN cells and a proportion of SP cells.

Analysis of fetal thymocyte development in Shh-and Gli3-deficient embryos has demonstrated that Shh provides a positive signal for thymocyte differentiation and proliferation at the DN1 to DN2 transition (Shah et al., 2004; Hager-Theodorides et al., 2005) (Figure 4). Thymi from Shh\(^{-/-}\) mice contained approximately 10 times fewer thymocytes than thymi from control littermates, and had reduced thymocyte proliferation and a partial arrest in development at the DN1 stage (Shah et al., 2004). There was a partial thymocyte block at the DN1 stage in Gli3\(^{-/-}\) thymi, but thymocyte number and proliferation was unaffected, suggesting that Gli3 is downstream of Shh in signaling for...
differentiation only (Hager-Theodorides et al., 2005). Using Smo knockouts, a role of Shh signaling in differentiation of early DN cells in adult thymi has been established (Andaloussi et al., 2006). Specifically, Shh signaling is critical for DN1 to differentiate to the DN2 stage. Analysis of Shh-/- mouse embryos has suggested that Shh influences very early thymocyte development prior to the DN1 to DN2 transition (Shah et al., 2004). The Shh-/- thymi of mice on embryonic day 13.5 were smaller and contained a reduced proportion of cells of hematopoietic origin, suggesting that Shh is necessary for their development either before or during entry of thymocyte progenitors to the thymus (Shah et al., 2004). The function of Hh signaling at the transition from DN to DP cells is controversial due to conflicting data which suggests that Hh can have a positive role (Shah et al., 2004), a negative role (Outram et al., 2000), or no role at all during this transition (Andaloussi et al., 2006). The most likely model is one that takes all data into account, in which Shh signals positively for thymocyte development prior to pre-TCR transduction, but acts as an inhibitor from the DN3 to DP transition after the pre-TCR signal (Crompton et al., 2007). Since Shh is a morphogen, it produces certain outcomes depending on the concentration and duration of the signal received, as well as the competence of the signal-receiving cell, so it is possible that Shh can act as either a positive or negative regulator based upon its concentration. For example, in Shh-/- fetal thymic organ cultures (FTOC), treatment with low-dose recombinant Shh increased DP cell production, while treatment with higher concentrations of recombinant Shh arrested differentiation (Shah et al., 2004).

Activation of the Shh signaling pathway in developing thymocytes influences TCR repertoire selection and the differentiation from DP to SP cells by influencing the
strength of the TCR signal through a mechanism upstream of extracellular-signal-regulated kinase (ERK) activation (Rowbotham et al., 2007a). In Shh\(^{-/-}\) FTOCs, the ratio of mature CD4\(^{+}\) to CD8\(^{+}\) cells was increased, which is consistent with an increase in TCR-signal strength during positive selection (Rowbotham et al., 2007a). When wild-type E17.5 FTOCs were treated with recombinant Shh, the ratio of mature CD4\(^{+}\) to CD8\(^{+}\) cells was decreased (Rowbotham et al., 2007a). In summation, studies on Shh’s role in the thymus demonstrate the necessity of Shh in thymocyte development and T cell activation.

**Wnt signaling**

The Wnt pathway derives its name from the Drosophila Wingless gene and the mouse INT-1 gene. Wnt genes encode a large family of secreted glycoproteins that are involved in a variety of cell activities in development (Miller, 2002). The importance of this pathway is quite evident, as mutations in the Wnt pathway or its components can result in a variety of diseases (Logan and Nusse, 2004) (Table 2). In humans, 19 WNT genes have been identified and the chromosomal locations of each are known (Coudreuse and Korswagen, 2007). With a few exceptions (Wg, Wnt3/5, and Wnt4), Wnt proteins are usually around 350 amino acids long and have an approximate molecular weight of 40 kDa (Miller, 2002). Wnts begin as precursors containing an N-terminal hydrophobic signal peptide that directs the
immature protein to the endoplasmic reticulum (ER) (Figure 5). In the ER, the signal peptide is cleaved by a protease and the Wnt protein is modified by addition of sugars and lipids by the acyl-transferase porcupine in order for proper secretion to occur. The attachment of a palmitate moiety to a conserved cysteine residue on the Wnts, which convert them into hydrophobic proteins, is essential for their biological activity (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)/Evenness interrupted (Evi) (Ching and Nusse, 2006).

**Table 2. Human genetic diseases caused by mutations in the Wnt pathway**

<table>
<thead>
<tr>
<th>Gene mutated</th>
<th>Disease</th>
<th>Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt3</td>
<td>Tetra-amelia</td>
<td>Absence of limbs</td>
</tr>
<tr>
<td>LRP5</td>
<td>Osteoporosis-pseudoglioma syndrome, familial exudative vitreoretinopathy</td>
<td>Bone density defects, Retinal angiogenesis defects</td>
</tr>
<tr>
<td>FZD4</td>
<td>Familial exudative vitreoretinopathy</td>
<td>Retinal angiogenesis defects</td>
</tr>
<tr>
<td>Axin2</td>
<td>Tooth agenesis, predisposition to colorectal cancer</td>
<td>Absence of multiple permanent teeth</td>
</tr>
<tr>
<td>APC</td>
<td>Polyposis coli, colon cancer</td>
<td>Polyps in colon and rectum</td>
</tr>
</tbody>
</table>

Adapted from Logan and Nusse (2004)

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 (Figure 6). The canonical pathway is stimulated by Wnt proteins that bind to cell surface Frizzled (Fz) receptors and the co-receptor low density lipoprotein receptor-related protein 5 (LRP5) or LRP6 (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 enters the nucleus, engages with LEF-1 and the TCFs, TCF1, TCF3, TCF4, and functions as a transcription factor (Novak and Dedhar, 1999). TCF and LEF are transcription factors that are bound to DNA and, in the absence of β-catenin, interact with transcriptional corepressors, such as Groucho, which inhibits transcription (Roose et al., 1998). Other proteins in the nucleus that control Wnt signaling events include Chibby, which is a nuclear antagonist that binds to the C-terminus of β-catenin (Takemaru et al., 2003), and Inhibitor of β-catenin and TCF (ICAT) (Tago et al., 2000), which blocks the binding of β-catenin to TCF (Tago et al., 2000), but also leads to dissociation of β-catenin and LEF complexes (Daniels and Weis, 2005). Activation of the Wnt target gene program is achieved when β-catenin displaces the Groucho/Transducin-like Enhancer of Split (TLE) corepressor proteins from Tcf (Daniels and Weis, 2005) and efficiently recruits a variety of proteins capable of effecting changes in local chromatin structure to the Wnt target genes (Stadeli et al., 2006). Many of these co-activator proteins, such as the histone acetylase cyclic-AMP-responsive-element binding protein (CREB)-binding protein (CBP), Brahma-related gene (BRG)-1, and Hyrax interact directly with the C-terminus of β-catenin. Another protein, Pygopus, indirectly binds the N-terminus of β-catenin via a common binding partner, Bcl9 (legless in Drosophila). The exact role of the β-catenin/Bcl9/Pygopus complex is controversial, in that one line of evidence suggests that it facilitates the nuclear import/retention of β-catenin (Townsley et al., 2004), while another study supports a direct role for the complex in enhancing the ability of β-catenin to activate Wnt target genes (Hoffmans et al., 2005). When Wnt signaling is absent, free β-catenin is bound by a destruction complex consisting of the tumor-suppressor gene products axis inhibitor (AXIN) and
adenomatous polyposis coli (APC), and the serine/threonine kinases casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β) (Behrens et al., 1996). After binding AXIN and APC, β-catenin is phosphorylated by CK1 and GSK3β in a sequential manner on at least four conserved N-terminal serine and threonine residues. CK1 phosphorylates β-catenin at Ser45, creating a priming site for GSK3β to subsequently phosphorylate the remaining residues, Thr41, Ser37, and Ser33. This creates a recognition motif for an E3-ubiquitin-ligase complex that contains β-transducin repeat containing protein (β-TRCP), which tags β-catenin with ubiquitin molecules, targeting it for proteosomal degradation (Ikeda et al., 1998; Amit et al., 2002; Gao et al., 2002). In contrast, when Wnt proteins bind to their receptors, formation of the Frizzled-LRP5/LRP6 complex through DVL (mammalian homologue of Drosophila Dishevelled) promotes the phosphorylation of LRP5 or LRP6 by CK1 and GSK3β (Zeng et al., 2008). Phosphorylation of LRP5 or LRP6 allows docking of AXIN1 thru the phosphorylated LRP residues, which removes AXIN1 from the destruction complex in the cytoplasm (Zeng et al., 2005). The recruitment of AXIN1 to the plasma membrane leads to inhibition of the phosphorylating ability of GSK3β and the destruction complex. This results in the escape of β-catenin and its movement to the nucleus. Relocation of β-catenin from the cytoplasm to the nucleus following its Wnt-induced stabilization is necessary for achieving efficient activation of Wnt target genes and ensuring appropriate physiological response. The way in which this is achieved has not been determined. These genes have not been well-defined in the hematopoietic system, with the exception of immature thymocytes, in which proliferation, cell adhesion, and anti-apoptotic genes have been identified as Wnt targets (Staal et al., 2004).
TECs are the main source of Wnt proteins in the thymus, while the Wnt receptor Fz is mostly found on thymocytes, which indicates a crosstalk between TECs and thymocytes (Pongracz et al., 2003). Wnt signaling occurs in the thymus and is most active in the immature DN stages (Weerkamp et al., 2006) (Figure 4). The first report to identify a functional role for Wnt signaling in thymocyte differentiation used retroviruses encoding the extracellular Wnt-binding domains of Fz receptors as extracellular inhibitors of Wnt signaling. Fetal liver stem cells were transduced with these soluble Fz receptors and subsequently assessed for their ability to develop into T cells using FTOC (Staal et al., 2001). These experiments showed a complete block of early thymocyte development, similar to that seen in $Tcf1^{Δ5/Δ5}Lef1^{-/-}$ double-knockout mice. This finding indicated that secreted Wnt factors are essential for intrathymic T lineage development. In addition, the direct effects of Wnt factors on fetal thymocytes...
were observed in an in vitro culture system (Staal et al., 2001). Specifically, expression of Wnt1 and Wnt4 through retroviral transduction resulted in increased numbers of cultured thymocytes in the absence of thymic stroma, likely attributed to a combined effect on survival and proliferation (Staal et al., 2001). Therefore, Wnt proteins act as growth factors for early thymocytes. The aforementioned findings were supported by in vivo studies that demonstrated reduced cellularity in the thymus due to reduced proliferation of cells mainly at the DN and ISP stages of development in Wnt1−/−Wnt4−/− mice (Mulroy et al., 2002). Studies have also shown that defects in T cell development occur in mice deficient for TCF1. Young TCF1-deficient mice have an incomplete block at the DN1, DN2, and ISP stages of thymocyte development, whereas older mice have a complete block most likely at the DN1 stage (Verbeek et al., 1995).

Several studies have shown that Wnt signaling is important at the DN to DP transition of thymocyte development. Expression of ICAT (Pongracz et al., 2006), which inhibits Wnt signaling by preventing binding of β-catenin to TCF and LEF factors, blocks this transition, but does not affect later stages of development. The secreted Wnt inhibitor Dikkopf-1 (DKK1), which blocks binding of Wnt to the required LRP co-receptor, inhibits thymocyte differentiation at the DN stage in a dose-dependent manner, such that high levels of DKK1 lead to complete inhibition of T-cell development at the DN1 stage (Weerkamp et al., 2006). Activation of the Wnt pathway by overexpressing activated forms of β-catenin resulted in the generation of more thymocytes (Mulroy et al., 2003), lack of requirement for pre-TCR signals in mice lacking pre-TCR (Gounari et al., 2001; Goux et al., 2005), and activated proliferation-associated genes in immature thymocytes (Staal et al., 2004). Conditional T-cell-
specific deletion of β-catenin, using the proximal lymphocyte protein tyrosine kinase (LCK) promoter to control Cre expression, impaired T-cell development at the TCR β-selection checkpoint, contributing to a marked decrease in the number of peripheral T cells (Xu et al., 2003). In addition, conditional deletion of the APC tumor-suppressor gene, which is part of the β-catenin destruction complex, also disrupts T cell development in the thymus, partly due to its effect on β-catenin signaling (Gounari et al., 2001).

Some studies have demonstrated a role for Wnt signaling in DP to SP thymocyte transition, although Wnt/β-catenin signaling is less active in signaling at this stage. Expression of CD4 by DP thymocytes is regulated partially by β-catenin-TCF signaling (Huang et al., 2006), and TCF1-deficient mice express lower levels of CD4 on DP and CD4⁺ SP cells. A series of studies showed that overexpressing stabilized β-catenin regulates the positive selection of thymocytes (Yu and Sen, 2007; Yu et al., 2007); the generation of fully selected CD4⁺ and CD8⁺ SP thymocytes occurred simultaneously when stabilized β-catenin was overexpressed, in contrast to the normal situation when generation of CD8⁺ cells occurs less frequently than generation of CD4⁺ cells. Collectively, previous studies have demonstrated a role for Wnt/β-catenin signaling in DN proliferation and differentiation, DP survival, and CD8⁺ generation.
Cadmium

Human exposure

Cadmium (Cd), a heavy metal, is an environmental pollutant due to its widespread and continual use. It currently ranks seventh on the Agency for Toxic Substances and Disease Registry/Environmental Protection Agency (ATSDR/EPA) list of Hazardous Substances (ATSDR, 2007). Cd levels in the environment vary widely due to its ability to be transported through air, water, and soil. Humans normally absorb Cd into the body either by ingestion or inhalation (Lauwerys et al., 1986). The daily intake of Cd 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 has a half-life of 15-20 years in humans, which contributes to its toxicity (Jin et al., 1998). The heavy metal's toxicity is contributed mostly to its interaction with essential nutrients, such as zinc (Zn), iron (Fe), calcium (Ca), and copper (Cu) (Flora et al., 2008) (Figure 7). Cd has no physiological function in humans, thus, excessive accumulation in the body often results in diseases and occasionally death (Othumpangat et al., 2005).

Exposure to the heavy metal and its compounds primarily occurs in workplaces such as mining, smelting, processing, and battery manufacturing, whereas non-occupational exposures come from various foods, contaminated water, and tobacco smoke. Natural sources of Cd that put Cd into the air include volcanic activity, forest fires, and windblown transport of soil particles. Food is usually the largest source of non-occupational exposure for non-smokers. Contamination of soil caused by mining,
application of phosphate fertilizers, and atmospheric deposition of airborne Cd leads to increased uptake by crops and vegetables grown for human consumption. Foods that have high concentrations of Cd include mollusks and crustaceans, offal products such as kidney and liver, oil seeds, cocoa beans, and certain wild mushrooms. In addition, cereals such as rice and wheat, green leafy vegetables, potatoes, and root vegetables contain a higher concentration of Cd than other plant foods. Based on estimation of Cd intake, more than 80% of Cd from food comes from cereals, vegetables, and potatoes (Olsson et al., 2002). Cd in drinking water contributes only to less than a few percent of the total Cd intake (Olsson et al., 2002). In heavily contaminated areas, however, such as those in the vicinity of Cd emitting plants or in mining districts, both well water and rivers may be substantially contaminated by Cd.

Figure 7. Cadmium interacts with essential nutrients

Adapted from Flora et al. (2008)
**Absorption and excretion**

Cd absorption from the gastrointestinal tract is the main route of Cd exposure in non-smoking humans. Approximately 3-10% of ingested Cd is absorbed from the gastrointestinal system, while 50% of Cd in inhaled smoke is absorbed through the bloodstream (Sahmoun et al., 2005). Smokers generally have Cd blood levels 4-5 times those of non-smokers (Elinder et al., 1976).

In animal studies, the fraction of Cd that is absorbed from the gastrointestinal tract is low, but increases with dose (Lehman and Klaassen, 1986). Once Cd is absorbed, it is transported into the liver, bound to albumin (Nordberg et al., 1992), where it induces the synthesis of metallothionein (MT). MT is a small molecular weight, cysteine-rich stress response protein that binds heavy metals with high affinity (Hamer, 1986). MT’s high affinity for metals allows for protection against Cd toxicity as well as maintaining the homeostasis of certain essential metals in mammals (Klaassen et al., 1999b). The primary site for MT synthesis is the liver (Coyle et al., 1995; Quaife et al., 1999); however, other cells such as lymphocytes and monocytes as well as lymphoid tissues such as the thymus can produce MT in response to particular stimuli (Olafson, 1985; Coto et al., 1992; Leibbrandt and Koropatnick, 1994; Yurkow and Makhijani, 1998). Following release from the liver, MT-bound Cd enters the plasma. MT-bound Cd emerges in the glomerular filtrate, from where it is reabsorbed intracellularly by renal tubule cells. While in the renal tubule cells, Cd is cleaved from MT by lysosomal action, and Cd\(^{2+}\) ions are re-excreted into the tubular fluid. Cd is eliminated in the urine, however, the amount excreted only represents 0.005-0.01% of the total body burden (Friberg, 1986).
Numerous studies have been conducted to determine the function of MT in Cd toxicology (Klaassen et al., 1999a). MT is easily induced by Cd and various metal ions, as well as by other stimuli (Kagi and Schaffer, 1988). Cd toxicity can be ameliorated by various MT inducers, particularly Zn (Klaassen et al., 1999a; Waalkes, 2003). Although MT plays a limited role in the initial distribution of Cd to various tissues (Liu et al., 1996), the retention of Cd in various tissues is MT-dependent. Cd mainly accumulates in kidney and liver, where high MT levels are found. Induction of hepatic MT abolishes biliary excretion of Cd (Klaassen, 1978), and renal Cd concentration is proportional to renal MT levels (Liu et al., 1996; Jarup et al., 1998). Molecules other than MT, such as albumin, cysteine, glutathione (GSH), and sulfydryl-rich proteins, can also form associations with Cd.

Cd uptake is mediated by transport proteins such as divalent metal transport 1 (DMT1), metal transport protein1, calcium channel proteins, and the 8-transmembrane zinc-related iron-related protein (ZIP8) to reach the target tissues. Alteration of transport protein expression can affect cellular Cd uptake and accumulation, and as a result, impact Cd toxicity (Zalups and Ahmad, 2003; Dalton et al., 2005; Leslie et al., 2006). For example, a diet low in iron increases the expression of the DMT1, which transports iron. DMT1 also transports Cd, thus low iron stores can increase Cd absorption, possibly contributing to toxicity.

The finding that pretreatment of animals with a low dose of Cd makes animals highly tolerant to Cd-induced lethality first demonstrated the role of MT in Cd toxicity (Goering and Klaassen, 1983). In wild-type and MT-null mice administered increasing doses of Cd, wild-type mice acquired tolerance to Cd lethality, with a 7-fold difference in
LD_{50} values, while MT-null mice did not display such tolerance (Park et al., 2001), indicating a critical role for MT as a major protein in protecting acute Cd poisoning. The liver is the major target organ of toxicity following acute Cd poisoning, and Cd hepatotoxicity is the major cause of acute Cd lethality (Goering and Klaassen, 1983). Acquired tolerance to acute Cd hepatotoxicity depends on the presynthesized MT in the liver, which functions to sequester Cd in the cytosol, which in turn, reduces the amount of Cd available for other critical organelles (Goering and Klaassen, 1983). The liver, spleen, and bone are important hematopoietic organs, that are all targets of Cd exposure (Klaassen et al., 1999a). Therefore, mice deficient in MT are likely more sensitive than wild-type mice to Cd-induced anemia and immunotoxicity (Liu et al., 1999). Blood MT is non-protective, however, low to moderate levels of blood MT has effects on the severity of autoimmune disease and on the development of adaptive immune functions (Lynes and Yin, 2006). Mice deficient in MT exhibited a significantly higher humoral response to challenge with ovalbumin compared to wild-type controls. Overall circulatory immunoglobulin levels are also significantly higher in MT-null mice than in wild-type mice (Crowthers et al., 2000).

Toxicology and Carcinogenicity

Itai-Itai disease

Public awareness of the toxic effects of Cd began with the post World-War-II (1950) outbreak in Toyama Prefecture, Japan, of “itai-itai” disease, which was the first Cd poisoning in the world. Itai-itai, or “ouch ouch” disease, so named due to the severe pain in the joints and spine, was caused by ingestion of runoff water containing Cd.
released from mining companies in surrounding mountains (Kagawa, 1994). Farmers used the contaminated water to irrigate crops, such as rice paddies. Cd concentrated in those crops, and when people ingested them, particularly women, they experienced excruciating pain. Cd interfered with calcium (Ca) metabolism leading to reduction in Ca levels, which reduced density and strength of bones, often leading to bone breakage.

**Reproductive effects**

Oocyte development and associated events have been disrupted by Cd administration in numerous different species, including *Xenopus laevis* (Lienesch *et al.*, 2000; Fort *et al.*, 2001; Fort *et al.*, 2002), rats (Paksy *et al.*, 1989; Paksy *et al.*, 1992; Piasek and Laskey, 1994; Paksy *et al.*, 1997b), mice (Rehm and Waalkes, 1988), hamsters (Saksena and Salmonsen, 1983; Rehm and Waalkes, 1988), pigs (Vrsanska *et al.*, 2003; Mlynarcikova *et al.*, 2005), and sheep (Leoni *et al.*, 2002). Exposure to Cd of cultured human ovarian granulosa cells was found to cause reduction in progesterone production when compared with controls, and also caused morphological alterations in a time-and dose-dependent manner, with rounding up of cells after 4-8 h, retraction of cellular extensions, and detachment from neighboring cells (Paksy *et al.*, 1997a). Testicular changes due to Cd toxicity have been seen in a variety of animal models at different stages of growth and maturity. Gonadal development in mouse embryos exposed to Cd in early organogenesis was studied by Tam and Liu (1985). Genital ridge size was reduced in exposed animals, with retarded germ cell migration into the ridges, resulting in depleted populations of germ cells, defective maturation of
gametes and subfertility in male offspring (Tam and Liu, 1985). Also, adult male rats have been shown to develop gonadal damage following administration of Cd, either orally or subcutaneously (SC). Focal testicular necrosis and reduced spermatogenesis were seen in rats that received a single dose of 100-150 mg Cd/kg orally within 2 weeks of administration (Kotsonis and Klaassen, 1977). Rats injected with a large dose of CdCl₂ (7 mg/kg SC) showed pronounced testicular hemorrhage and edema 24 h after treatment (El-Ashmawy and Youssef, 1999). In addition, heavy smoking has been associated with low sperm count and motility. A strong positive relationship has been identified between smoking, serum and seminal fluid Cd levels in infertile Nigerian males (Akinloye et al., 2006). Other studies, however, have shown no differences in semen quality or fertility between smokers and non-smokers, even though significantly increased amounts of Cd were found in seminal fluid in those smoking 20 or more cigarettes a day (Saaranen et al., 1989). In the rat model, increased rates of abnormal sperm morphology have been noted with increased seminal Cd levels, with positive correlation with TNF-α and IFN-γ, and a negative relationship with IL-4 (Al-Bader et al., 1999). All seminal parameters and cytokine differences in this model were reversible by administration of Zn with Cd. It has been suggested that absence of certain micronutrients in the diet contribute to male infertility associated with increased Cd levels (Akinloye et al., 2006).

Cd may also reduce the possibility of a successful pregnancy by interfering with the development of the pre-implantation embryo. Gamete fusion in the ovine model was found to be significantly reduced following exposure to 2-20 µM Cd (Leoni et al., 2002). Fertilization of mouse oocytes was unaffected when cultured at 1.6 µM Cd, and
treated ova cleaved into two-cell stage embryos at a comparable rate to controls, although a reduced number of these embryos reached blastocyst stage (Schmid et al., 1983). In vitro studies in which murine embryos were cultured at higher Cd concentrations (10-50 µM) at the two-cell stage, demonstrated a dose-dependent inhibition of developmental progression (Storeng and Jonsen, 1980). In vivo exposure of the murine embryo to Cd at the two-cell stage by SC injection of the dam with 25-38 µM Cd/kg body weight on the morning of day 2 (D2) failed to prevent initiation and maintenance of pregnancy when examined on D8, but did delay implantation (De et al., 1993). Embryos that implanted went on to become normal fetuses, with resorption rates and body weights comparable to controls (De et al., 1993). Studies have shown that Cd inhibits gap junction, intercellular communication, and connexin phosphorylation (Jeong et al., 2000; Fang et al., 2001), all of which are necessary for progression from the eight-cell stage through to compaction (Hardy et al., 1996; Kidder and Winterhager, 2001). Protection of the early embryo from Cd toxicity has been achieved by preventing Cd uptake by cells by pre- or co-incubation with Zn at 100x molar dose of Cd (Yu and Chan, 1988; De et al., 1993) and by co-culture with nifedipine (Ca²⁺ channel blocker) at 0.01x molar dose of Cd (De et al., 1993). Antioxidants are also known to be protective against Cd toxicity (Peters et al., 1995).

The presence of MT in the placenta is generally accepted to be a protective mechanism against Cd toxicity within the cells in which it is produced, and is also thought to restrict Cd movement across the placenta, as the level of Cd is lower in fetal than in maternal blood (Goyer and Cherian, 1992). In contrast, some (Goyer and Cherian, 1992; Torreblanca et al., 1992) think MT has detrimental effects, by binding Zn.
or by interfering with the ability of trophoblast cells to handle Ca and Zn at the cytosolic level. Using the perfused human placenta, Wier et al. (1990) demonstrated a reduction in transplacental Zn transfer to the fetus, and also a drop in human chorionic gonadotrophin (hCG) production by the placenta, beginning 4 h after exposure. Placentas of smoking mothers contained twice the Cd and approximately half the progesterone of those of non-smokers. Piasek et al found a reduction in placental iron in smokers with elevated levels of placenta Cd (Piasek et al., 2001). Cd interference with progesterone biosynthesis in cultured human trophoblast cells has been linked with reduced activity of P450 cholesterol side-chain cleavage enzyme and decreased 3β-hydroxysteroid dehydrogenase enzyme mRNA (Kawai et al., 2002).

**Teratogenicity**

Cd is known to exert teratogenic and embryotoxic effects in many species as confirmed by animal studies (Webster and Messerle, 1980; Messerle and Webster, 1982; Menoud and Schowing, 1987; De et al., 1990; Sunderman et al., 1992). The changes induced are dependent on the strain and species studied, the dose of Cd administered, the route of administration, and the stage of embryogenesis (Layton and Layton, 1979; Feuston and Scott, 1985; Holt and Webb, 1987; De et al., 1990). Toxic renal effects in 2- to 60-day-old postnatal offspring were observed when pregnant rats were administered CdCl\(_2\) (0.5 mg/kg\(^{-1}\)/day) orally throughout gestation (Jacquillet et al., 2007). When pregnant rats were treated with 0.3 or 0.6 mg/kg\(^{-1}\) Cd subcutaneously from E7 to E15, sensorimotor development delays were observed in the offspring (Minetti and Reale, 2006). In the hamster, Gale and Layton (1980) injected a single
dose of 2 mg/kg CdSO$_4$ intravenously on E8, and found numerous craniofacial, skeletal, and soft tissue abnormalities upon examination of fetuses on E15. When a whole-embryo culture assay was used for hamster embryos, a 70% abnormality rate in embryos cultured at 0.25 mM Cd on E8 was observed, with animals exhibiting growth retardation, reduction in somite gain, axial anomaly and neural tube defect (Wlodarczyk et al., 2001). Cd has also been shown to have teratogenic effects on limb development. Following administration of CdSO$_4$ to C57Bl/6 mice at day 9.5 of gestation, a high incidence of postaxial forelimb ectrodactyly in offspring resulted due to inhibition of the anterior/posterior formation process (Scott et al., 2005). This process is controlled by Shh signaling.

Cd has not been shown to be teratogenic in humans; however, an inverse relationship has been found between birth weight and cord blood Cd levels (Galicia-Garcia et al., 1997), birth weight and placental Cd concentration (Ronco et al., 2005), and between birth length and maternal blood Cd (Nishijo et al., 2004). There is an association between maternal Cd exposure and early delivery, which may serve as an explanation for smaller babies born to women who smoke during pregnancy (Nishijo et al., 2002). An inverse relationship has also been demonstrated between birth weight and Cd content in hair of newborns (Huel et al., 1981; Frery et al., 1993). Cd blood levels in newborn infants have been found to be 70% of maternal levels (Lagerkvist et al., 1992). The difference in Cd level can most likely be attributed to placental function, as perfusion of human placentas with Cd showed that transfer rate from maternal to fetus was very slow (Boadi et al., 1991). Cd did not appear on the fetal side of the placenta until 40 minutes (min) after perfusion started, and reached a steady state
approximately 1 hour (h) later, at which time the concentration in the fetal perfusate was 1/20 that of the maternal.

Postnatal effects due to abnormally high levels of Cd during gestation include significantly increased DNA synthesis in the bowel and bone marrow in 6-week old rats following administration of Cd orally to pregnant dams at a dose of 50 parts per million (ppm) in drinking water (Konecki et al., 2000), and depressed immune function and sensorimotor abilities in children (Schoeters et al., 2006). Human studies are not conclusive, so additional work must be done to determine the extent and type of damage to children following prenatal exposure.

Immunotoxicity

There have been numerous studies on the immunomodulatory effect of Cd in humans and experimental animals; however, the findings remain controversial (Descotes, 1992). Dan et al. (2000) showed that Cd causes significant suppression of humoral and cell-mediated immune response in mice, and suggested that this effect may be due to its cytotoxic action on liver, kidney, and immune cells. There have also been reports that Cd induces immunostimulation in experimental animals (Koller and Roan, 1977). This conflict amongst findings may be attributed to varying doses, route of administration, length of Cd treatment, and sensitivity of immune systems between different animal species. Strain specific immune responses to Cd may be due to variation of Cd binding to MT and tissue storage or possibly because strains differ in their proliferative response level to polyclonal T cell mitogens (Dan et al., 2000). The thymus is a target organ of Cd-induced toxicity. Following Cd-treatment, damage to the
thymus as well as changes in the proliferation rate of thymocytes in rats results (Morselt et al., 1988). In addition, several in vivo experiments 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 supported these findings and demonstrated that Cd exposure increased the number of DN cells (2007).

Cd exposure results in modulation of antibody production, inhibition of B-cell activation, enhancement of mortality in mice challenged with streptococci, and decreased mortality of influenza-infected mice (Koller et al., 1976; Gardner et al., 1977; Fujimaki et al., 1982; Chaumard et al., 1983; Ohsawa et al., 1986; Daum et al., 1993). In vitro exposure to 10 µM Cd induced apoptotic features in mouse thymocytes (Fujimaki et al., 2000). A study demonstrated that Cd (0-100 ppm), when given to rats in drinking water, showed a differential effect on the blood lymphocyte phenotyping (Lafuente et al., 2004). Evidence suggests the mechanism of Cd toxicity is attributed to oxidative stress, i.e. increased lipid peroxidation, reactive oxygen species (ROS) generation, and alterations in GSH and related enzymes, under both in vitro and in vivo conditions (Stohs and Bagchi, 1995; Hart et al., 1999; Thevenod et al., 2000; de la Fuente et al., 2002). The role of ROS in Cd induced apoptosis (Pulido and Parrish, 2003; Lemarie et al., 2004) is further supported by activation of redox sensitive AP-1 transcription factor and alteration in GSH metabolism prior to apoptosis (Hart et al., 1999). Immune cells are highly sensitive to oxidative stress due to major production of ROS (Meydani et al., 1995). ROS plays a major role in immune function by acting as intracellular signals. Also, oxidant-antioxidant balance is important for maintenance of
the immune response (Kim et al., 2001). The apoptogenic potential of Cd was demonstrated in murine thymocytes involving mitochondrial membrane depolarization, and caspase activation, and ROS and GSH acting as critical mediators (Pathak and Khandelwal, 2006). Differential effects of Cd on the spleen and thymus have been demonstrated (Pathak and Khandelwal, 2007b). Suppression of cell proliferative response, thymic atrophy, and splenomegaly following Cd exposure may be due to oxidative stress followed by activation of the mitochondrial-caspase dependent apoptotic pathway (Pathak and Khandelwal, 2007b). Differential susceptibility of various T subsets to the apoptogenic effects of Cd in the order of CD8+>DN>CD4+>DP was reported (Dong et al., 2001). In addition, Cd treatment affected cell surface marker expression and led to phenotypic changes, characterized by a decline in DP cells and a marked lowering in CD4+/CD8+ ratio mainly due to a significant increase in CD8+ subsets (Dong et al., 2001). The authors concluded that Cd was capable of inducing apoptosis in cultured mouse thymocytes in a dose and time-dependent manner (Dong et al., 2001). Another study examining the effect of in vitro exposure to Cd on thymocyte development showed that Cd significantly suppressed the proportion of CD4+ cells followed by DP (Pathak and Khandelwal, 2007a). The DN and CD8+ cells, on the other hand, were markedly enhanced (Pathak and Khandelwal, 2007a). In addition, cytokine analysis demonstrated an inhibition of IL-2 and IFN-γ release by Th1 cells, and IL-4 release by Th2 cells (Pathak and Khandelwal, 2007a). The authors attributed this to a lowered CD4+ population resulting in a decreased proportion of Th1 and Th2 cells, or to an indirect influence of the metal mediated by the endocrine system (Lafuente et al., 2004). Th1 and Th2 cells mediate cytotoxic and local inflammatory reactions, and
therefore play important roles in combating intracellular pathogens including viruses, bacteria, and parasites.

When adult male rats were exposed for one month to CdCl₂, Cd accumulated in the spleen (at ≥50 ppm), and in the thymus (at ≥ 10 ppm) (Lafuente et al., 2003). In both spleen and thymus, the B lymphocytes increased with doses of 5 and 10 ppm, and decreased with the doses of 25-100 ppm (Lafuente et al., 2003). In the spleen, doses of 25 and 50 ppm decreased CD4⁺ cells and the doses of 5 and 10 ppm increased CD8⁺ cells (Lafuente et al., 2003). In addition, results suggest that the spleen is more sensitive to Cd exposure than the thymus, although the accumulation of the metal was higher in the thymus than the spleen (Lafuente et al., 2003). Another study by Lafuente et al. (2004) demonstrated that Cd effects on the distribution of blood lymphocyte subsets suggest that Cd inhibits the humoral and cellular immune responses with lower doses (5-10 ppm), but opposite effects are demonstrated with higher doses (25-100 ppm). In a study that investigated the role of estradiol (E₂) on the immunotoxic effects of Cd in the rat, results indicated that Cd and E₂ not only share certain similar cellular pathways but also can interact to alter immune functions (Pillet et al., 2006). In addition, Cd can also modulate the hormonal balance and may exert estrogenic effects via interaction with estrogen receptors (ERs). This suggests that females may be at a greater risk than males for Cd-induced immunomodulation.

In immune and inflammatory responses, macrophages are activated (Auger, 1992) and secrete cytokines, ROS, reactive nitrogen species such as nitric oxide (NO) (Ding et al., 1988) and eicosanoids such as prostaglandin E₂ (PGE₂). These substances all contribute to tissue damage mediated by activated macrophages (Laskin
and Pendino, 1995). Both NO and PGE\textsubscript{2} are inflammatory and immunomodulatory mediators in mammalian physiology (Moncada \textit{et al.}, 1991; Reilly \textit{et al.}, 1998). Several oxidative stressors can induce the co-expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase type-2 (COX-2), which synthesizes NO and PGE\textsubscript{2}, respectively (Feng \textit{et al.}, 1995; Swierkosz \textit{et al.}, 1995; O’Banion, 1999). A study reported that in vitro Cd exposure in non-cytotoxic conditions induces iNOS expression (Ramirez \textit{et al.}, 1999) and enhances lipid peroxidation, arachidonic release, and GSH release from mouse peritoneal macrophages (Ramirez \textit{et al.}, 2001). Ramirez and Gimenez (2003) demonstrated that chronic exposure through drinking water (15 ppm for 2 months) induces redox changes in favor of pro-oxidant conditions, and increases synthesis of NO and PGE\textsubscript{2} in mouse peritoneal macrophages (Ramirez and Gimenez, 2003).

Neonatal exposure to environmentally relevant levels of Cd (10 ppb or 5 ppm) via maternal milk resulted in gender-specific delays in the development of females (Pillet \textit{et al.}, 2005). Also, exposure to Cd through maternal milk also produced transitory and persistent alterations of immune functions (Pillet \textit{et al.}, 2005). The immunotoxic effects were dependent on the type of immune cell, the developmental stage, and the level of exposure. Transitory and persistent alterations of the cytotoxic activity of NK-cells were only observed in males (Pillet \textit{et al.}, 2005). This was the first report of a gender-specific difference in the immunotoxic effects of Cd.

Exposure to Cd at sub-toxic levels has been documented to cause impairment of immunosurveillance, which controls expression and inactivation of viruses and bacteria. Significant depression in the phagocytic activity (Loose \textit{et al.}, 1978) as well as
suppression of humoral and cell-mediated immune responses (Dan et al., 2000) were also reported. It was found that susceptibility to bacteria in Cd-exposed mice was due to a defect of macrophage recruitment to sites of infection (Simonet et al., 1984). Two studies demonstrated that Cd increases susceptibility of affected individuals to bacterial and viral infections (Shen et al., 2001; Simonyte et al., 2003).

The effects of Cd on human cytokine responses, in particular T cell-derived cytokines, are fragmentary and contradictory. One study reported a significant depression of NK cell activity, but no changes in IFN-γ production by Cd treatment (Daniels et al., 1987). In contrast, another study demonstrated a significant enhancement in the IFN-γ production following occupational Cd exposure in men (Yucesoy et al., 1997). Hemdan et al. (2006) showed that human peripheral blood mononuclear cells exposure to low and moderate Cd doses (0.013-13.3 µM) resulted in a clear bias of the immune response toward Th1 or Th2 depending on the activation method applied (Hemdan et al., 2006). When the cells were activated with mononuclear antibodies (anti-CD3/anti-CD28/anti-CD40) and exposed to Cd acetate for 24 h, secretion of IL-1β, TNF-α, and IFN-γ was greatly inhibited compared to the production of IL-4 and IL-10, which indicates a Th2 biased immune response. When the cells were stimulated with bacterial antigen (heat-killed Salmonella Enteritidis) and exposed to Cd acetate for 24 h, secretion of IL-10 was highly suppressed compared to that of IFN-γ and TNF-α, while IL-4 was undetectable. These results imply that low Cd polarizes the immune response toward Th2 in cells stimulated via T cell receptor, while a polarized Th1 response is induced by bacterial antigens (Hemdan et al., 2006). In a study by Krocova et al. (2000), in vitro treatment of splenocytes from Balb/c mice with
Cd (20 µg/ml) demonstrated that Cd seemed to trigger the Th2 cytokine pathway (IL-4, IL-10). This effect of Cd may imply the possible autoimmune or allergic complications in individuals exposed to this heavy metal (Krocova et al., 2000).

Carcinogenic effects

The toxicology and carcinogenicity of Cd, and its environmental impacts have been well studied (Waalkes, 2000; Waalkes, 2003). There is sufficient evidence in humans to classify Cd and Cd compounds as carcinogens based on epidemiological studies demonstrating a link between Cd and lung, and possibly prostate, cancer (IARC, 2004). In addition, experimental animal studies demonstrate that Cd and Cd compounds by multiple routes of exposure induce benign and malignant tumor formation at various sites in many species of experimental animals (Waalkes et al., 1992a; Waalkes et al., 1992b; Waalkes and Rehm, 1994; Waalkes et al., 1999a; Waalkes et al., 1999b; Waalkes, 2000; Achanzar et al., 2001; Waalkes, 2003; Goyer et al., 2004). In rodents, Cd has induced tumors in several organs. Various Cd compounds produced adenocarcinomas of the lung in rats after inhalation (Takenaka et al., 1983). Tumors of the prostate and the pancreas were induced by subcutaneous injection of CdCl₂ in rats, tumors of the testis were observed following oral exposure of rats, and Cd produced local tumors at various sites of injection, typically sarcomas, in rats and mice (Waalkes, 2000). Cd administration that was linked with tumors of the prostate and testis in experimental animals was prevented by Zn administration (Nordberg et al., 1992). Although a positive relationship has been demonstrated
between proliferative testicular lesions and Cd in rats (Waalkes et al., 1999a; Waalkes et al., 1999b), no definitive link exists in humans.

The first studies to suggest a link between Cd and prostate cancer showed an increased risk of disease in occupationally exposed workers in a nickel-Cd battery factory (Potts, 1965; Kipling, 1967). Additional occupational studies demonstrated an association between Cd exposure and an increase in prostate cancer risk and mortality (Blair and Fraumeni, 1978; Dubrow and Wegman, 1984). More recent studies failed to show a link between occupational exposure to Cd and prostate cancer; however, studies have found that prostate cancer was associated with dietary exposure to Cd through drinking water and food (Bako et al., 1982; Elghany et al., 1990; West et al., 1991).

**Mechanisms of toxicity**

**Oxidative stress**

Cd, unlike other heavy metals, is unable to generate free radicals by itself; however, reports have indicated that superoxide radical, hydroxyl radical, and NO radicals could be generated indirectly (Galan et al., 2001). Cd can generate non-radical hydrogen peroxide, which by itself becomes a significant source of free radicals via Fenton chemistry (Watanabe et al., 2003). Cd could replace Fe and Cu from many cytoplasmic and membrane proteins like ferritin, which would then release and increase the concentration of unbound Fe^{2+} or Cu^{2+} ions. These free ions participate in causing oxidative stress via Fenton reactions (Casalino et al., 1997; Waisberg et al., 2003).
Numerous studies in animal models have shown that Cd intoxication significantly increased malondialdehyde and GSH peroxidase (Yang et al., 2003). Acute intoxication of animals with Cd has shown increased activity of antioxidant defense enzymes like Cu-Zn containing superoxide dismutase, catalase, GSH peroxidase, GSH reductase, and glutathione-S-transferase (Ognjanovic et al., 2003). Studies have shown that the number of cells with DNA single strand breaks and the levels of cellular DNA damage were significantly higher in Cd exposed animals. Reports have shown that antioxidants like vitamin C and vitamin E have demonstrated protection against Cd induced toxicity in different animal models (Ognjanovic et al., 2003). Supplementation of these natural antioxidants reduced ROS levels, lipid peroxidation, hematological values, and enzymatic and non-enzymatic components of antioxidant defense system.

Genotoxicity

The Cd$^{2+}$ ion easily substitutes for the Ca$^{2+}$ ion in biological systems, because it carries the same charge and has a similar radius. Compared to the Zn$^{2+}$ ion, the radius of the Cd$^{2+}$ ion is larger, but Cd$^{2+}$ ions can substitute for Zn$^{2+}$ ions in many enzymes and transcription factors. The genotoxicity of Cd is attributed to indirect mechanisms such as the generation of ROS, inhibition of DNA repair enzymes, and deregulation of cell proliferation. Cd has been shown to induce the formation of ROS, both in vitro and in vivo. CdS induced H$_2$O$_2$ formation in human polymononuclear leukocytes, and CdCl$_2$ increased production of superoxide in rat and human phagocytes (Sugiyama, 1994). In support of this, induction of DNA strand breaks and chromosomal aberrations by Cd in mammalian cells was suppressed by antioxidants and antioxidative enzymes (Ochi et
al., 1987; Stohs et al., 2001; Valko et al., 2006). The induction of oxidative stress by Cd is interpreted by its inhibitory effects on antioxidant enzymes such as catalase, superoxide dismutase, GSH reductase, and GSH peroxidase (Valko et al., 2006). Cd is considered co-mutagenic and exacerbates the mutagenicity of UV radiation, alkylation, and oxidation in mammalian cells (Hartwig and Schwerdtle, 2002). Cd inhibits several types of DNA repair such as base excision, nucleotide excision, mismatch repair, and the elimination of the premutagenic DNA precursor 8-oxo-dGTP (Hartwig and Schwerdtle, 2002). Low concentrations of Cd inhibited the repair of oxidative DNA damage in mammalian cells (Dally and Hartwig, 1997; Fatur et al., 2003). In regards to nucleotide excision repair, Cd interfered with the removal of thymine dimers after UV irradiation by inhibiting the first step of this repair pathway, which is the incision at the DNA lesion (Hartwig and Schwerdtle, 2002; Fatur et al., 2003). Cd disrupted the removal of 8-oxo-dGTP from the nucleotide pool by inhibiting the 8-oxo-dGTPases of bacterial and human origin (Bialkowski and Kasprzak, 1998). A molecular mechanism related to the inactivation of DNA repair proteins involves the displacement of Zn from Zn-finger proteins structures in the DNA repair proteins such as from the xeroderma pigmentosum group A (XPA) protein, which is required for nucleotide excision repair, and from the formamidopyrimidine [fapy]-DNA glycosylase (Fpg) protein, which is involved in base excision repair in E.coli (Asmuss et al., 2000). Cd induces a conformation shift in the Zn binding domain of the tumor suppressor protein p53 (Meplan et al., 1999). In addition to inhibiting repair proteins directly, Cd downregulates genes involved in DNA repair in vivo (Zhou et al., 2004). In Cd-adapted cells, the impairment of DNA repair by Cd may be especially harmful. Cd induces several genes
for Cd and ROS tolerance such as those coding for MT, GSH synthesis and function, catalase and superoxide dismutase (Stohs et al., 2001), which establishes an environment where prolonged cell survival in the presence of Cd occurs (Chubatsu et al., 1992).

Tolerance to Cd toxicity may constitute an extended opportunity for induction of further critical mutations (Achanzar et al., 2002). Cd interacts with a number of cellular signaling pathways, many of which are associated with mitogenic signaling. DNA synthesis and proliferation of rat myoblast cells (von Zglinicki et al., 1992) and rat macrophages (Misra et al., 2002) were stimulated by submicromolar concentrations of Cd. In various cell types in vitro, Cd induces receptor-mediated release of second messengers inositol-1,4,5-trisphosphate and Ca, it activates various mitogenic protein kinases, transcription, and translation factors, and it induces the expression of cellular proto-oncogenes c-fos, c-myc, and c-jun (Waisberg et al., 2003).

In addition to directly stimulating mitogenic signals, Cd also inhibits negative controls of cell proliferation, such as inactivating the tumor suppressor protein p53 and inhibiting the p53 response to damaged DNA (Meplan et al., 1999). Another mechanism by which Cd affects cell proliferation is through disruption of the WNT/β-catenin pathway resulting in dysregulation of cadherin-mediated cell-cell adhesion system and of cell-cell communication. Studies have shown that Cd perturbs the Wnt pathway in chick embryonic periderm (Thompson et al., 2008) and proximal tubule cells (Prozialeck et al., 2003; Thevenod et al., 2007).
Estrogenic effects

At a dose similar to the World Health Provisional Tolerable Weekly Intake, Cd mimics the in vivo effects of estrogen in target organs in animal studies (Johnson et al., 2003). Johnson et al. (2003) determined that, similar to estrogens, exposure of ovariectomized animals to a low dose of Cd (5 µg/kg body weight) results in the induction of estrogen target genes and an increase in uterine wet weight that are blocked by an antiestrogen. In the mammary gland, Cd also induces estrogen target genes and promotes the growth and differentiation that are blocked by an antiestrogen. In utero exposure to Cd mimics in utero exposure to estradiol (Johnson et al., 2003). When female offspring are exposed to Cd in utero, there is an accelerated onset of puberty and altered mammary gland development (Johnson et al., 2003). In support of a role for Cd in breast cancer, exposure to the metal has been linked to an increased risk of the disease in a hypothesis-generating case-control study that examined over 33,000 death certificates attributed to breast cancer and over 117,000 non-cancer deaths between 1984-1989 that were coded for occupation and industry (Cantor et al., 1995). The study found that Cd was associated with an approximate 8 to 20% increase in breast cancer risk among white women and 50-130% increase in risk among African-American women. Similar to estradiol, Cd activates ERα through the ligand binding domain. When COS-1 cells are transiently cotransfected with GAL-ER, a chimeric receptor containing the DNA binding domain of the transcription domain of ERα, a GAL4-responsive reporter gene, and treated with Cd, there is an increase in reporter gene activity that is blocked by an antiestrogen suggesting that Cd activates ERα through the ligand binding domain of the receptor (Stoica et al., 2000). It has been
reported that Cd modulates steroid hormone-dependent signaling in ovaries in rats, in a breast cancer cell line, and in Cd-transformed prostate epithelial cells (Benbrahim-Tallaa et al., 2007; Brama et al., 2007). However, in in vitro estrogenicity assays based on ER activity, no effect of Cd was detected (Silva et al., 2006).

**Androgenic effects**

The exact mechanism by which androgens affect prostate carcinogenesis remains to be elucidated; however, the hormones are strong promoters of the disease. The growth and development of the prostate gland is controlled by androgenic sex steroids. Androgens also play a central role in the development of prostate cancer. A causal relationship between androgens and prostate cancer is supported by the sensitivity and response of many prostate cancers to hormonal therapy (Waalkes and Rehm, 1994; Bosland, 2000). The LNCaP cells are an androgen dependent human prostate cancer cell line (Horoszewicz et al., 1980). In LNCaP cells, Cd mimics the effects of androgens on cell growth (Webber et al., 1985; Martin et al., 2002) and gene expression (Martin et al., 2002). Environmentally relevant doses of Cd mimic androgen effects in castrated rats and mice (Martin et al., 2002). Cd produces a dose-dependent increase in the wet weight of the prostate gland and the seminal vesicle complex that is blocked by an antiandrogen, which suggests that the metal activates the androgen receptor in target tissue (Martin et al., 2002). In intact animals, Cd addition to the drinking water significantly increases the weight of the prostate and seminal vesicles and the increase in weight was not reversed upon castration (Visser and Deklerk, 1978). The aforementioned data suggest that Cd is also a metalloandrogen and may
explain to some degree the heightened risk of prostate cancer associated with Cd exposure.
Industrial and environmental pollutants exert adverse effects on a number of organ systems in humans and animals. Cd, a heavy metal, is one such pollutant. Although there is evidence demonstrating that adult exposure to Cd causes changes in the immune system, there are limited reports of immunomodulatory effects of prenatal exposure to Cd. After combining studies that show prenatal Cd exposure causes perturbations in Shh and Wnt/β-catenin signaling, and these pathways play a role in thymocyte development, it is not difficult to postulate that prenatal exposure to Cd may affect thymocyte development via the Shh and Wnt/β-catenin pathways. Due to Cd’s long half-life, determining whether the effects of prenatal exposure to Cd at birth continue to adulthood is also vital to understanding Cd’s toxic mechanism. Since humans are exposed to Cd on a daily basis via the environment, and that pregnant women are more susceptible to Cd absorption than the rest of the population (Akesson et al., 2002), determining the effects that prenatal Cd exposure has on offspring will allow a better understanding of Cd’s toxic effect on the immune response. In addition, the findings may also elucidate the teratogenic mechanism of Cd in other organ systems that rely on Shh and Wnt/β-catenin signaling for proper development.

Summary

Industrial and environmental pollutants exert adverse effects on a number of organ systems in humans and animals. Cd, a heavy metal, is one such pollutant. Although there is evidence demonstrating that adult exposure to Cd causes changes in the immune system, there are limited reports of immunomodulatory effects of prenatal exposure to Cd. After combining studies that show prenatal Cd exposure causes perturbations in Shh and Wnt/β-catenin signaling, and these pathways play a role in thymocyte development, it is not difficult to postulate that prenatal exposure to Cd may affect thymocyte development via the Shh and Wnt/β-catenin pathways. Due to Cd’s long half-life, determining whether the effects of prenatal exposure to Cd at birth continue to adulthood is also vital to understanding Cd’s toxic mechanism. Since humans are exposed to Cd on a daily basis via the environment, and that pregnant women are more susceptible to Cd absorption than the rest of the population (Akesson et al., 2002), determining the effects that prenatal Cd exposure has on offspring will allow a better understanding of Cd’s toxic effect on the immune response. In addition, the findings may also elucidate the teratogenic mechanism of Cd in other organ systems that rely on Shh and Wnt/β-catenin signaling for proper development.
REFERENCES


Amit, S., Hatzubai, A., Birman, Y., Andersen, J. S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y., and Alkalay, I. (2002). Axin-mediated CKI phosphorylation of beta-


biological half time in kidney cortex, considering calorie intake and smoking habits. *Archives of environmental health* **31**, 292-302.


GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. The EMBO journal 17, 1371-1384.


from porcine follicles. *Physiological research / Academia Scientiarum Bohemoslovaca* 52, 383-387.


Wnt signaling in the thymus is regulated by differential expression of intracellular signaling molecules, pp. 3322-3326.


CHAPTER 2: Prenatal cadmium exposure dysregulates sonic hedgehog and Wnt/β-catenin signaling in the thymus resulting in altered thymocyte development
Prenatal cadmium exposure dysregulates sonic hedgehog and Wnt/β-catenin signaling in the thymus resulting in altered thymocyte development

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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; however, this was not due to altered Shh and Wnt protein levels. Target genes of Shh ( patched1 and gli1) and Wnt/β-catenin (c-fos, c-jun, 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.
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. The DN population is then 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. observed a decrease in DP cells (2001). Pathak and Khandelwal supported these findings and demonstrated that Cd exposure increased the number of DN cells (Pathak and Khandelwal, 2007).

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, 1996). Ptc releases its suppression of the cell surface molecule
Smoothened (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., 2001). 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-3β (GSK-3β) 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). 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 ddH$_2$O and filtered through Whatman no.1 paper. Samples were then diluted to volume in 10 ml ddH$_2$O. 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 min 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’-TTTGATGTCACGATTTCC-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 min at 94°C, then 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, then 5 min 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-PE (BD Biosciences Pharmingen). 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 x 10⁶) 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 mins 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 Figures 4 and 7, purified populations of thymocytes (DN, DP, CD4⁺, CD8⁺) were sorted by flow cytometry using the antibodies
listed above. 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 93%.

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 x 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 mAm per 18 h. Proteins were
transferred onto Hybond-P membranes (Amersham Pharmacia, Piscataway, NJ) at 0.5
amps for 3 h. 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-Wnt10b (Abcam, Cambridge, MA), anti-active-β-
catenin, clone 8E7 (Millipore, Temecula, CA), anti-phospho-β-catenin (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-HP 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 ± S.E.M. For Figures 1-3, 5, and 6A, a mean of the data from 3 mice/litter was taken and used as the N=1 data for the corresponding dam. For Figures 4, 6B, and 7, 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≤0.05 was considered significant. Relative gene expression data was analyzed using the $2^{\Delta\Delta C_T}$ method described in 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) µg/g kidney tissues, which demonstrates a low environmentally relevant exposure level that is 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

Thymocyte phenotype of representative offspring from each litter was measured by cell surface marker expression using flow cytometry. Total thymocyte number was not significantly different between Cd-treated and control offspring (7.74±0.53 x 10^6 vs. 8.75±0.63 x 10^6, respectively). The number of CD4+ cells was significantly increased in Cd-treated offspring compared to control offspring [3.09±0.44 x 10^6 vs 1.48±0.21 x 10^6 (p<0.05), respectively] (Figure 1A). The number of CD8+ cells showed a decreasing trend in Cd-treated offspring, though the difference was not significant (p<0.06). 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 showed that Cd-treated offspring had significantly more DN4 cells (3.64±0.12 x 10^5)
compared to control offspring (1.93±0.08 x 10^5) (p<0.01) (Figure 1C). The DN3 population also showed an increasing trend in Cd-treated offspring, though the difference was not significant (p<0.06). 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 2). Cyclopamine (5µM), 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 3A). 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 3B). 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 4A). 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.
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 5A). 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 5B). 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 protein levels, translational and transcriptional levels of Wnt10b were determined by western blot and real-time RT-PCR, respectively. 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 protein in thymic lysates showed
comparable levels between Cd-treated and control offspring (Figure 6A). 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 6B). Protein levels of Wnt10b protein in thymocytes could not be detected by western blot, thus the Wnt10b that was detected in whole thymic lysates was from TECs.

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 7A). Analysis of c-jun showed no significant difference between Cd-treated and control offspring (Figure 7B). 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 7C). In summary, prenatal Cd exposure increased at least one Wnt/β-catenin target gene in all thymocyte subpopulations analyzed except CD8+ thymocytes.
Figure 1

A

Control Cd-treated

\[
\begin{align*}
\text{Number of Cells (x10^6)} \\
\text{DN} & \quad \text{DP} & \quad \text{CD4} & \quad \text{CD8} \\
\end{align*}
\]

B

\[
\begin{align*}
\text{CD4/CD8 Ratio} \\
\text{Control} & \quad \text{Cd-treated} \\
\end{align*}
\]

C

\[
\begin{align*}
\text{Number of Cells (x10^5)} \\
\text{DN1} & \quad \text{DN2} & \quad \text{DN3} & \quad \text{DN4} \\
\end{align*}
\]
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. Each bar represents the mean ± SEM. Data is representative of 3 independent experiments where N=6 in each group. (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

Relative luciferase activity

Control  Cd-treated
Figure 2. Shh signaling activity in thymic lysates of PND0 offspring. Thymic lysates were prepared from newborn mice (< 12 h old) whose mothers were exposed to 10 ppm Cd throughout pregnancy. Shh signaling was measured by luciferase activity in Shh-Light 2 cells. Each bar represents the mean ± 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
Figure 3

A

Control Cd-treated

GAPDH

Shh/GAPDH

0.00
0.05
0.10
0.15
0.20
0.25
0.30

GAPDH

Shh

Cd

B

Cd

Gli1/GAPDH

0.00
0.05
0.10
0.15
0.20
0.25
0.30

GAPDH

Cd

Gli1

Control Cd-treated
Figure 3. Shh and Gli1 expression in thymic lysates of PND0 offspring. Thymic lysates were prepared from newborn mice (< 12 h old) whose mothers were exposed to 10 ppm Cd throughout pregnancy. 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. The ratio of N-Shh protein to GAPDH protein (A) and the ratio of Gli1 protein to GAPDH protein (B) was determined using densitometry of the detected bands.
Figure 4

A

Relative expression of Gli1 mRNA

- Control
- Cd-treated

DN DP CD4 CD8

B

Relative expression of Ptc mRNA

DN DP CD4 CD8
Figure 4. Hh target gene expression in thymocyte subpopulations of PND0 offspring. Primers specific for (A) Gli1 and (B) 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. *, $\geq$ 3-fold difference between control and Cd-treated groups.
Figure 5

A

Cd

ABC

Oct-1

0 1 2 3 4

Control Cd-treated

ABC/Oct-1

* p-β-catenin

GAPDH

B

Cd

p-β-catenin

GAPDH

0.0 0.1 0.2 0.3 0.4 0.5 0.6

phospho-β-catenin/GAPDH

Control Cd-treated
Figure 5. 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
Figure 6. **Wnt10b expression in thymus of PND0 offspring.** (A) 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. (B) 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.
Figure 7

A

Relative expression of c-fos mRNA

DN | DP | CD4 | CD8

Control | Cd-treated

B

Relative expression of c-jun mRNA

DN | DP | CD4 | CD8

C

Relative expression of c-myc mRNA

DN | DP | CD4 | CD8
Figure 7. Wnt/β-catenin target gene expression in thymocyte subpopulations of PND0 offspring. Primers specific for (A) c-fos, (B) c-jun, and (C) 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.
A

Thymic epithelium

Shh precursor molecule

autocleavage

cholesterol

Shh-N

N- -COOH

Shh-C

N- -COOH

Shh-Np

N- -COOH

palmitate

Dispatched

Wnt10b

multimerization

Shh-Np

Thymocytes

cytoplasm

nucleus

Gli1

DN

↑Gli1

↑Ptc1

DP

Gli1

↑Gli1

Ptc1

↑Gli1

Ptc1

CD4

CD8

B

Thymic epithelium

ER

Porcupine

Golgi

palmitate

Wntless/Evi

Wnt

Wnt

Wnt10b

palmitate

Thymocytes

cytoplasm

nucleus

phospho-β-catenin

DN

c-fos

↑c-fos

c-myc

c-jun

↑c-myc

c-jun

DP

c-fos

↑c-fos

c-myc

c-jun

↑c-myc

c-jun

CD4

c-fos

↑c-fos

c-myc

c-jun

c-myc

c-jun

CD8

c-fos

↑c-fos

c-myc

c-jun

c-myc

c-jun
Figure 8. 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.
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; Pathak and Khandelwal, 2007), an increase in DN cells (Pathak and Khandelwal, 2007), and a decrease in the CD4⁺/CD8⁺ ratio (Dong et al., 2001; Pathak and Khandelwal, 2007). In those studies, primary thymocytes from 3-6 week old male Balb/c mice were exposed in vitro to various Cd concentrations (5-50 μM) for several time intervals (3-24 h). However, the relevance of these studies to the interpretation of those reported herein is questionable because of the differences in experimental design such as in vitro vs. 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. Although Cd was detected in pooled livers of the offspring, the level was close to the minimum level of detection, thus we believe that any possible direct Cd effect is minimal to nil. 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 Shh signaling showed that prenatal Cd exposure dysregulates several components of this pathway (Figure 8A). 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 8A, top). The downregulation of Shh target gene Wnt10b in the whole thymus further supports an inhibitory role of prenatal Cd exposure on Shh signaling from the epithelium. Analysis of Shh protein in thymic lysates via western blot indicates that active Shh protein levels are unchanged. 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 cholesterol modification of the active 19 kDa fragment (Porter et al., 1995), palmitoylation of the N-terminal cysteine (Pepinsky et al., 1998), requirement of the
protein Dispatched for secretion (Burke et al., 1999), and multimerization of the Shh protein (Zeng et al., 2001). Since the antibody used in this study recognizes the cholesterol-modified form of Shh, it is unlikely that Cd affects this step of modification; however, any of the other steps 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 8A, bottom). Based on our data, 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 thymocyte phenotype showed a significant increase in the DN4 cell population as well as a trended increase in the DN3 cell population, thus, increased Shh signaling may contribute to increased proliferation of these cell populations. The role that Shh signaling plays in single positive cells has not been well defined, however, one study showed that Shh signaling in developing thymocytes
influences TCR repertoire selection and differentiation from DP to SP cells (Rowbotham et al., 2007). Specifically, in Shh−/− fetal thymic organ cultures, the CD4+/CD8+ ratio was increased, therefore, the decreased Shh signal from TECs in the present study is likely contributing to the increased CD4+/CD8+ ratio observed in prenatal Cd-treated offspring. In addition, the increased transcription of Ptc1 and Gli1 in CD8+ and no change in these genes in CD4+ cells may also contribute to the significantly increased CD4+/CD8+ ratio observed in thymocyte phenotype analysis.

Analysis of Wnt/β-catenin signaling showed that prenatal Cd exposure dysregulates several components of this pathway (Figure 8B). Wnt proteins begin as precursors containing an N-terminal hydrophobic signal peptide that directs the immature protein to the endoplasmic reticulum (ER). In the ER, the signal peptide is cleaved by a protease and the Wnt protein is modified by the addition of sugars and lipids by the acyl-transferase porcupine (Tanaka, 2000). This is required for proper secretion to occur. The attachment of a palmitate moiety to a conserved cysteine residue on the Wnts, which converts them into hydrophobic proteins, is essential for their biological activity (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)/Evenness interrupted (Evi) (Ching and Nusse, 2006). TECs are the main source of Wnt proteins in the thymus, while the Wnt receptor, Frizzled (Fz), is mostly found on thymocytes, which indicates a crosstalk between TECs and thymocytes (Pongracz et al., 2003). In our study, we observed unchanged protein levels of Wnt10b in TECs (Figure 8B, top), along with decreased Wnt/β-catenin signaling in thymocytes as determined by increased cytoplasmic phospho-β-catenin and decreased nuclear
ABC (Figure 8B, bottom). These findings suggest that prenatal Cd treatment is affecting the palmitoylation processing or secretion steps of Wnt in the epithelium. The decrease in properly processed/secrated 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 8B, 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 trended decrease following prenatal Cd exposure. Although c-fos, c-jun, 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 resulting in aberrant thymocyte development. It is likely that prenatal Cd exposure affects the processing and/or secretion of Shh and Wnt proteins in the thymic epithelium resulting in an attenuated signal in both pathways as demonstrated by a decrease in Gli:luciferase activity and a decrease in nuclear ABC levels. 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. In addition to the thymus, Hh and Wnt/β-catenin signaling is necessary for proper development of the kidney (Stark et al., 1994; Kang et al., 1997), bone (Gao et al., 2001; Gong et al., 2001; Loughlin et al., 2004), lung (Pepicelli et al., 1998; Tebar et al., 2001; Mucenski et al., 2003; Okubo and Hogan, 2004), and prostate (Podlasek et al., 1999; Truica et al., 2000; Lamm et al., 2002; Mulholland et al., 2002; Chesire and Isaacs, 2003; Freestone et al., 2003; Berman et al., 2004), all of which are target organs of Cd toxicity (Bernard, 2008). 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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REFERENCES


Tang, N., and Enger, M. D. (1993). Cd(2+)-induced c-myc mRNA accumulation in NRK-49F cells is blocked by the protein kinase inhibitor H7 but not by HA1004, indicating that protein kinase C is a mediator of the response. *Toxicology* **81**, 155-164.


CHAPTER 3: Prenatal cadmium exposure alters postnatal immune cell development and function
Prenatal cadmium exposure alters postnatal immune cell development and function

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Running title: Prenatal cadmium affects immune cells

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ABSTRACT

Cadmium (Cd) is an environmental pollutant due to its widespread and continual use. Cd is generally found in low concentrations in the environment, while its concentration in cigarette smoke is high. 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. This study was designed to investigate the effects of prenatal exposure to Cd on the immune system of the offspring. Pregnant C57Bl/6 mice were exposed to an environmentally relevant dose of Cd (10 ppm) and the effects on the immune system of the offspring were assessed at two time points following birth (PND 14 and 49). Thymocyte and splenocyte phenotypes were analyzed by flow cytometry. Cytokine production of splenic T cells was determined by ELISA. Prenatal Cd exposure increased the number of double negative (DN; CD4^+CD8^-) thymocytes, specifically the DN1 subpopulation (CD44^+CD25^-), at PND 14 and 49. In the spleen, prenatal Cd exposure decreased the number of macrophages in male offspring at both time points. Cytokine analysis of splenic T cells demonstrated that prenatal Cd exposure decreased IL-2 and IL-4 production in female offspring at PND14. At PND49, IL-2 production remained decreased in Cd-exposed females while IFN-γ production was decreased in both male and female Cd-treated offspring. These findings suggest that even a very low level of exposure to Cd during gestation can result in long term detrimental effects on the immune system of the offspring, and these effects are to some extent sex-specific.

Key words: cadmium, prenatal exposure, thymocytes, splenocytes, cytokines
INTRODUCTION

Cadmium (Cd) is a heavy metal that poses a hazard to human health due to its toxicity. There is sufficient evidence in humans to classify Cd and Cd compounds as carcinogens based on epidemiological studies demonstrating a link between Cd and lung, and possibly prostate, cancer (IARC, 2004). Exposure to the heavy metal and its compounds primarily occurs in workplaces such as mining, smelting, processing, and battery manufacturing, whereas non-occupational exposures come from various foods, contaminated water, and tobacco smoke. Smokers generally have Cd blood levels 4-5 times those of non-smokers (Elinder et al., 1976).

Cd levels in the environment vary widely due to its ability to be transported through air, water, and soil. 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). The average Cd intake from food generally varies between 8-25 µg per day (Olsson et al., 2002), 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).

As a result of humans not having an effective Cd elimination pathway, the biologic half-life of Cd in the body is estimated to be 15-20 years (Jin et al., 1998). Excessive Cd accumulation in the body often results in diseases such as kidney failure, respiratory disease, neurological disorders, and occasionally death (Waalkes et al., 1992). Although pharmacokinetic studies have demonstrated that Cd does not readily reach the fetus, it accumulates in high concentrations in the placenta (Piasek et al.,
2001). Teratological effects associated with Cd exposure reported for humans are limited; however, maternal exposure to environmental Cd, higher placental concentration (Loiacono et al., 1992), and/or fetal Cd exposure (Frery et al., 1993) has been associated with lower birth weights in humans. Moreover, the teratological effects of Cd in rodents have been extensively documented (Hovland et al., 1999; Scott et al., 2005; Minetti and Reale, 2006; Jacquillet et al., 2007).

There have been numerous studies on the immunomodulatory effects of Cd in humans and experimental animals; however, the findings remain controversial (Descotes, 1992). This conflict amongst findings may be attributed to varying doses, route of administration, length of Cd treatment, and sensitivity of immune systems between different animal species. The thymus, the primary site of T-cell production, is a target organ of Cd-induced toxicity (Morselt et al., 1988). 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. The DN population can be 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 the CD44^−CD25^−(DN4) population. Following Cd-treatment, damage to the thymus as well as changes in the proliferation rate of thymocytes in adult rats results (Morselt et al., 1988). In adult mice, Dong (2001) observed a decrease in DP cells. Pathak and Khandelwal (2007a) also demonstrated that Cd exposure decreased the DP population and increased the number of DN cells.
In vivo studies exposing adult male rats to varying concentrations of Cd (0-100 ppm) demonstrated that lower doses of Cd inhibited humoral and cellular immune responses, while higher concentrations had a stimulatory effect (Lafuente et al., 2004). Analysis of oxidative stress and apoptosis showed that splenic cells appeared more susceptible than thymus cells to the adverse effects of Cd both in vitro (Pathak and Khandelwal, 2006a; Pathak and Khandelwal, 2006b) and in vivo (Pathak and Khandelwal, 2007b).

Despite the numerous studies demonstrating the effects of Cd on the adult immune system, there have been limited reports on the effect of Cd exposure during gestation on the immune system of the offspring. We have previously shown that prenatal exposure to Cd affects thymocyte development of newborn (<12 h old) offspring (Hanson et al., unpublished). This study was designed to investigate the continued effects of prenatal exposure to Cd on the immune system of the offspring. Pregnant mice were exposed to an environmentally relevant dose of Cd (10 ppm) and the effects on the immune system of the offspring were assessed at two time points following birth [post-natal day (PND) 14 and 49] to evaluate the effects in relation to developmental stage. In addition, whether the observed prenatal effects on the immune system were sex-specific was determined.
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. 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). Cd administration was stopped at birth. At PND14 and 49, 3 offspring (at least 1 male and 1 female) selected from each of the litters were euthanized and thymi and spleens were removed. Mice born from a different set of dams were euthanized at PND49 to ensure adequate sample size. The PND14 time point was chosen to determine if any longer term effects of prenatal exposure to Cd were evident early in life and PND49 was chosen to assess the effects of prenatal Cd exposure at the mouse adult stage. This time point would approximate a younger post-pubescent human which should have a fully developed robust immune response. All offspring were weaned at PND21 and the dams were euthanized.
**Tissue Isolation and Cell Preparation**

Thymi and spleens 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.

**Cd load**

A group of dams whose litters were not used in the present study, but were dosed in the same manner, were euthanized following parturition to determine Cd load. To measure Cd content, kidney samples were dissolved in 2 ml of 70% nitric acid. The acidified samples were neutralized in 5 ml of ddH$_2$O and filtered through Whatman no.1 paper. Samples were then diluted to volume with ddH$_2$O in a 25 ml volumetric flask. 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. The purpose of this assay was to determine how much Cd was retained in the kidneys of the dams to verify that the dams were consuming approximately equal amounts of water, and thus, Cd dosing was consistent between dams.

**Cell staining and flow cytometry**

Single cell suspensions of thymocytes and splenocytes 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-PE (BD Biosciences Pharmingen). 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. Splenocytes were stained using combinations of the following directly conjugated Abs: anti-CD4-FITC (BD Biosciences Pharmingen), anti-CD8-PE (BD Biosciences Pharmingen), anti-F4/80-APC-Alexa Fluor 750 (eBioscience), anti-Gr1-Alexa Fluor 700 (eBioscience), and anti-B220-PE-Cy5 (BD Biosciences Pharmingen). Cells (1 x 10⁶) 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 min on ice to block Fc receptors, followed by a PBSAz wash. The cells were incubated for 30 min 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 FACSArria and FACSDiva software (BD Biosciences Pharmingen). A total of 10,000 events were collected for each sample.

**Cytokine production**

The release of Interleukin-2 (IL-2), Interleukin 4 (IL-4), Interleukin 10 (IL-10), and Interferon-gamma (IFN-γ) in splenocytes at PND14 and PND49 was measured using the Mouse TH1/TH2 Ready-SET-Go! ELISA set (eBioscience). Briefly, 1 x 10⁶ cells/ml
in RPMI media (Mediatech Cellgro; Manassas, VA) supplemented with 10% FBS were seeded in 48-well plates and stimulated with anti-CD3 (10 µg/ml; eBioscience) and anti-CD28 (10 µg/ml; eBioscience). The culture supernatant was collected 24 h after the stimulation for the assessment of IL-2, and 72 h after the stimulation for the assessment of IL-4, IL-10, and IFN-γ. The concentrations of the cytokines were assessed per manufacturer’s protocol.

**Statistical Analysis**

Results are expressed as mean ± S.E.M. For Figure 1, a mean of the data from 3 mice/litter was taken and used as the N=1 data for the corresponding dam. For Figures 2-4, a mean of the data from 5-8 offspring/sex/treatment group/age group was taken. Statistical analyses comparing the values for a particular cell population or cytokine concentration 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. All experiments were repeated at least three times.
RESULTS

Tissue Cd levels

The average Cd concentration for the dams was 4.37±0.76 (SEM) µg/g kidney tissues, which demonstrates a low environmentally relevant exposure level that is consistent between dams. Analysis of Cd levels in the offspring were attempted; however no Cd was detected in their 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 PND14 and 49 offspring

Thymocyte phenotype of representative offspring from each litter was measured by cell surface marker expression using flow cytometry. Total thymocyte number was not significantly different between Cd-treated and control offspring at PND14 (25.9±3.4 x 10^7 vs. 23.0±3.0 x 10^7, respectively) or at PND49 (25.4±2.2 x 10^7 vs. 21.2±1.8 x 10^7, respectively). At PND14, the number of DN cells was significantly increased in Cd-treated offspring compared to the control offspring [1.95±0.28 x 10^7 vs. 1.34±0.20 x 10^7 (p<0.05), respectively] (Figure 1A). The significant increase in the DN population persisted until PND49 [Cd-treated offspring, 2.29±0.21 x 10^7 DN cells vs. control offspring, 1.80±0.12 x 10^7 DN cells (p<0.05)]. No other population of thymocytes was significantly altered at either day of analysis. Analysis of subpopulations of the DN population at PND14 showed that Cd-treated offspring had significantly more DN1 cells...
(4.86±0.97 x10^6) compared to control offspring (2.80±0.41 x 10^6) (p<0.05) (Figure 1B).

The increase in DN1 cell number among Cd-treated offspring was still present at PND49 [Cd-treated, 9.93±0.90 x 10^6 vs. control, 7.72±0.53 x 10^6 (p<0.05)]. The DN2 cell numbers also showed an increasing trend in Cd-treated offspring at PND14 and 49, though the difference was not significant (p<0.06). There were no sex-specific effects on thymocyte phenotype observed in PND14 or PND49 offspring.

**Effect of prenatal Cd exposure on splenocyte phenotype in PND14 and 49 offspring**

Splenocyte phenotype of representative offspring from each litter was determined by flow cytometry using cell markers specific for CD4 and CD8 T cells, B cells, macrophages, and granulocytes. Total splenocyte number was not significantly different between Cd-treated and control offspring (1.1±0.8 x 10^8 vs. 1.0±0.93 x 10^8, respectively). The only cell type that showed a significant difference between the Cd-treated offspring and control animals was macrophage (Figure 2). Further, this difference only occurred in male offspring. At PND14, Cd-treated males had 0.78±0.27 x 10^7 macrophages while control offspring had 1.47±0.14 x 10^7 (p<0.05) macrophages (Figure 2A). The significant decrease in Cd-treated males was still present at PND49 [(Cd-treated, 1.35±0.22 x 10^7 vs. control, 2.25±0.27 x10^7 (p<0.05)] (Figure 2B). All other cell populations had no significant differences between the two treatment groups.
Effect of prenatal Cd exposure on spleen cell ex vivo cytokine production at PND14 and 49

In order to determine the effects of prenatal Cd exposure on the immune response of offspring at PND14 and 49, production of IL-2, IL-4, IL-10, and IFN-γ by splenic T cells was measured following stimulation with anti-CD3/28. At PND14, Cd-treated females produced significantly less IL-2 than control females [Cd-treated, 2.31±0.77 µg/ml vs. control, 7.40±1.41 µg/ml (p<0.01)] (Figure 3). IL-4 production was also significantly decreased in Cd-treated females compared to control females [Cd-treated, 202.42±7.15 pg/ml vs. control, 299.76±17.86 pg/ml (p<0.05)] (Figure 3). Cd-treated male offspring did not demonstrate any statistically significant difference in cytokine production at PND14, although IFN-γ was markedly decreased in Cd-treated male offspring (p<0.06). At PND49, IL-2 production remained significantly decreased in Cd-treated females compared to control females [Cd-treated, 3.36±1.02 µg/ml vs. control, 8.21±1.22 µg/ml (p<0.05)] (Figure 4). IFN-γ was significantly decreased in both Cd-treated males (Cd treated, 0.47±0.10 µg/ml vs. control, 12.52±2.48 µg/ml) and females (Cd-treated, 3.69±0.98 µg/ml vs. control, 9.29±2.54 µg/ml) compared to control animals (p<0.05) (Figure 4). There were no differences in IL-10 or IL-4 production in either Cd-treated males or females at PND49. In addition, the cytokines Interleukin-12p70 (IL-12p70), Tumor Necrosis Factor-α (TNF-α), Monocyte Chemoattractant Protein-1 (MCP-1), and Interleukin-6 (IL-6) were measured but none of these cytokines showed any difference between the Cd-treated offspring and the controls (data not shown). Taken together, cytokine production by prenatally Cd-treated female offspring is affected at an earlier developmental stage than their male counterparts. In female
offspring at PND49, the data suggest that the Th1 cells (IL-2 and IFN-γ producing) are affected, while the Th2 cells (IL-10 and IL-4 producing) are not. In the male offspring at the same time point, there was no effect on the IL-2, IL-4, or IL-10 production, yet IFN-γ was significantly decreased.
Figure 1

A

Number of DN Cells (x10^7)

Number of Cells (x10^7)

DN  DP  CD4  CD8

* PND14-Control
* PND14-Cd-treated
* PND49-Control
* PND49-Cd-treated

B

Number of Cells (x10^6)

DN1  DN2  DN3  DN4

*
Figure 1. Thymocyte phenotype of PND14 and 49 offspring. Thymocytes were isolated from 14 and 49 day old mice that were exposed to 10 ppm Cd in utero. Single cell suspensions were prepared for flow cytometry analysis by standard methods. Each bar represents the mean ± SEM. Data are representative of 3 experiments where N=4 in each group. (A) Thymocyte phenotype was determined based on CD4 and CD8 cell surface expression. (B) DN subpopulation phenotype was determined based on CD44 and CD25 cell surface expression. *p<0.05
Figure 2

A

B

Control males
Cd-treated males
Control females
Cd-treated females

Number of Cells (x10^7)

>10^4

CD4+ CD8+ F4/80+ Gr-1 B220+

Number of Cells (x10^7)

CD4+ CD8+ F4/80+ Gr-1 B220+
Figure 2. Splenocyte phenotype of PND14 and 49 offspring. Spleen cells were isolated from 14 (A) and 49 (B) day old mice that were exposed to 10 ppm Cd in utero. Single cell suspensions were prepared for flow cytometry analysis by standard methods. Flow cytometric analysis was performed by staining splenocytes with anti-CD4, anti-CD8, anti-B220 (B cells), anti-F4/80 (macrophages), and anti-Gr-1 (granulocytes). Each bar represents the mean ± SEM. Data are representative of 3 experiments where N=5-8 offspring/sex/treatment group/age group. *p<0.01
Figure 3

![Graph showing cytokine concentrations](image_url)

- IL-2
- IFN-γ
- IL-10
- IL-4

**p < 0.05,  
**p < 0.01
Figure 3. Cytokine expression of splenic T cells on PND14. Spleen cells were isolated from 14 day old mice that were exposed to 10 ppm Cd in utero. Cells were stimulated with anti-CD3/CD28 for 24h (IL-2) or 72h (IFN-γ, IL-10 and IL-4). Supernatants were analyzed for cytokine expression by ELISA kits. Limit of detection: IL-2 (2 pg/ml), IFN-γ (15 pg/ml), IL-10 (30 pg/ml), IL-4 (4 pg/ml). Each bar represents the mean ± SEM. Data are representative of 3 experiments where N=5-8 offspring/sex/treatment group. *p<0.05, **p<0.01
Figure 4
Figure 4. Cytokine expression of splenic T cells on PND49. Spleen cells were isolated from 49 day old mice that were exposed to 10 ppm Cd in utero. Cells were stimulated with anti-CD3/CD28 for 24h (IL-2) or 72h (IFN-γ, IL-10 and IL-4). Supernatants were analyzed for cytokine expression by ELISA kits. Limit of detection: IL-2 (2 pg/ml), IFN-γ, (15 pg/ml), IL-10 (30 pg/ml), IL-4 (4 pg/ml). Each bar represents the mean ± SEM. Data are representative of 3 experiments where N=5-8 offspring/sex/treatment group. *p<0.05
DISCUSSION

Immunotoxicity after Cd exposure in adult animals is well documented; however, reports concerning the effect of Cd exposure during gestation on the immune system are limited. We have previously demonstrated that prenatal Cd exposure alters thymocyte development in offspring at PND0 (Hanson et al, unpublished), while another study showed that proliferative responses of spleen cells to mitogens and activity of peritoneal macrophages were increased, and delayed type hypersensitivity to sheep red blood cells after immunization was decreased, in prenatal Cd-exposed offspring (Soukupova et al., 1991). Due to organogenesis of the immune system occurring mostly at the prenatal, and to a lesser extent, at the early postnatal stage, the perinatal period is not only more sensitive to deleterious effects of immunotoxicants, but alterations in the immune system can result in persistent effects (Holladay and Smialowicz, 2000). Exposure to toxic agents such as halogenated aromatic hydrocarbons (HAHs), polycyclic aromatic hydrocarbons (PAHs), hormonal substances, and heavy metals during the developmental period can result in a range of functional defects in adulthood, including suppression of the immune system (Blaylock et al., 1992; Keil et al., 2008), hypersensitivity (Miller et al., 1998), and autoimmune disease (Snyder et al., 2000; Mustafa et al., 2008). In vitro studies have demonstrated that Cd causes oxidative stress and apoptosis in adult mouse T- and B-cells (Pathak and Khandelwal, 2006b; Pathak and Khandelwal, 2006a), as well as macrophages (Kim and Sharma, 2006). The thymus is an important primary lymphoid organ where successive stages of cell development and selection produce functionally competent T cells from
immature precursor cells. Several in vivo studies demonstrated that adult exposure to Cd is able to cause significant weight decrease or atrophy of the thymus in mice using a wide range of doses (Borgman et al., 1986; Mackova et al., 1996; Liu et al., 1999). In addition, in vitro studies have demonstrated that exposure to Cd results in apoptosis and phenotypic changes in thymocytes from adult mice (Dong et al., 2001; Pathak and Khandelwal, 2007a). The present analysis of offspring thymocyte phenotype following prenatal Cd exposure demonstrates an increase in the DN population, which is in agreement with previous reports of Dong (2001) and Pathak (2007a). Examination of the four DN subpopulations demonstrates that the earliest stage in thymocyte development, DN1, was significantly increased after prenatal Cd exposure at both PND14 and 49. We have previously shown a link between prenatal Cd exposure and dysregulation of the sonic hedgehog (Shh) and Wnt/β-catenin signaling pathways in the thymus at PND0 (Hanson et al, unpublished). Both of these pathways are needed for differentiation of DN1 cells to DN2 cells, therefore a continued perturbation of these pathways at PND14 and 49 may be responsible for the altered thymocyte phenotype.

In vitro and in vivo studies in adult mice have shown that splenocytes are more sensitive to Cd treatment than are thymocytes (Pathak and Khandelwal, 2006b; Pathak and Khandelwal, 2006a; Pathak and Khandelwal, 2007b). Although we did not observe a significant decrease in the total number of splenocytes in prenatally Cd-treated offspring, an analysis of splenocyte phenotype demonstrated that the macrophage population was decreased in Cd-treated offspring. More strikingly, this effect was only present in male offspring. In vivo studies by others indicate that chronic Cd exposure alters the redox balance in adult male mice, inducing changes in lipid metabolism in
macrophages, ultimately leading to apoptosis (Ramirez and Gimenez, 2002). In addition to being antigen presenting cells and phagocytes, macrophages can recognize tumor cells and induce cell death by releasing cytotoxic factors such as reactive oxygen and/or nitrogen intermediates, as well as cytokines; thus, the decrease in macrophage number in prenatal Cd-treated male offspring may increase susceptibility of males to tumor incidence. Further studies will have to be conducted to understand the mechanism of this cell type-, sex-specific effect.

Since cytokines influence or control most immune responses, cytokine production by splenic T cells of offspring following prenatal Cd exposure was determined. In vitro studies have suggested that Th1 type cytokines (IL-2, IFN-γ) are depressed to a higher degree than Th2 type cytokines (IL-10, IL-4) following Cd treatment (Krocova et al., 2000; Hemdan et al., 2006; Pathak and Khandelwal, 2007a). Studies demonstrating that a decrease in Shh signaling in peripheral CD4+ T cells down-regulates the synthesis of IL-2 and IFN-γ (Stewart et al., 2002) and that prenatal Cd exposure decreases Shh signaling in the thymus of offspring at PND0 (Hanson et al., unpublished), led to the hypothesis that Th1 cells in the spleen would be more sensitive to prenatal Cd exposure than Th2 cells. Analysis at PND14 demonstrated that cytokine production (IL-2 and IL-4) was only decreased in females. Analysis at PND49 revealed a dramatic Th1 type (IL-2 and IFN-γ) cytokine decrease by T cells from female offspring, as well as a decrease in IFN-γ secretion by T cells from male offspring. The IL-4 levels at PND14 reached comparable levels to control females by PND49, thus this cytokine may be affected in early development but returns to normal levels as the immune system reaches maturity. Mouse studies evaluating sex differences following Cd
exposure are sparse. In humans, although no studies have been designed to investigate sex differences directly, related studies have shown that Cd-associated health effects are more frequent among women than men (Vahter et al., 2002). This may be due to a higher Cd body burden in women, reflected as higher Cd levels in blood, urine, and kidney cortex (Vahter et al., 2007). The main reason for the higher body burden in women is increased intestinal absorption of dietary Cd at low iron stores (Akesson et al., 2002; Kippler et al., 2007). Cd and iron compete with one another for transport into the mucosa cell via the divalent metal transporter 1 (DMT-1). When a woman is pregnant, enterocytes have an increased DMT-1 density at the apical surface to increase absorption of micronutrients, thus Cd absorption is increased during pregnancy (Akesson et al., 2002; Vahter et al., 2007). It has been reported that 90% of patients with Itai-Itai disease, the most severe form of chronic Cd intoxication in humans, were postmenopausal women (Jarup et al., 1998). This is due to Cd’s ability to disrupt calcium homeostasis caused by estrogen depletion during menopause or following ovariectomy (Jarup et al., 1998). Another reason for sex-differences in susceptibility to Cd-induced toxicity may be attributed to Cd having estrogenic effects (Garcia-Morales et al., 1994; Stoica et al., 2000; Sogawa et al., 2001; Choe et al., 2003; Johnson et al., 2003). Following in utero exposure to Cd, female offspring experienced an earlier onset of puberty and an increase in the epithelial area and the number of terminal end buds in the mammary gland (Johnson et al., 2003). In addition, in vivo studies using adult female rats suggest that females may be at a greater risk than males for Cd-induced immunomodulation due to interactions between estrogen and Cd (Pillet et al., 2006). Pathak and Khandelwal (Pathak and Khandelwal, 2007a) demonstrated
that IFN-γ is inhibited at a lower Cd concentration than IL-2 in adult male mice, so if female offspring have a higher body burden or are more sensitive to prenatal Cd treatment, then a higher dose of Cd may be necessary to elicit a decrease in IL-2 in male offspring, thus explaining the lack of effect on IL-2 production, but decreased IFN-γ production in male offspring in the present study.

The immunomodulatory effects of prenatal Cd exposure observed in this study may provide insight as to why children of women who smoke during pregnancy have an increased risk for developing cancer later in life. Several studies have indicated that smoking while pregnant increases the risk of certain types of childhood cancers, such as leukemias, lymphomas, and central nervous system tumors, in the prenatally exposed offspring (Filippini et al., 1994; Filippini et al., 2000; Schuz et al., 2001; Brooks et al., 2004). In the U.S., more than a million newborns are exposed to cigarette smoke during gestation (DHHS, 2000). A single cigarette typically contains 1-2 µg of Cd. When burned, Cd is present at a level of 1,000-3,000 ppb in the smoke (ATSDR, 1999). Approximately 40 to 60% of the Cd inhaled from cigarette smoke is able to pass through the lungs and into the body (Sahmoun et al., 2005). This means that for each pack of cigarettes smoked, a person can absorb an additional 1-3 µg of Cd in addition to the amount taken in from other sources in one’s daily life. Assuming that adult mice drink approximately 3 ml of water/day, the concentration of Cd being ingested by mice in the present experiment is nearly equivalent to the concentration of Cd that would be inhaled from smoking 1 pack of cigarettes/day. A study in which pregnant mice were exposed to a low concentration of mainstream cigarette smoke throughout gestation increased the incidence and growth rate of EL-4 induced tumors in male offspring, as well as
reduced cytotoxic T lymphocyte activity in male offspring at 5 and 10 weeks after birth (Ng et al., 2006). Ng et al. (2006) findings support epidemiologic data indicating that children of mothers who smoke during pregnancy have a greater risk of developing cancer later in life. The decreased macrophage number, as well as decreased IFN-γ production in male offspring in the present study may explain the increased susceptibility to tumor incidence and growth in male offspring exposed to cigarette smoke in utero.

In summary, we have demonstrated that prenatal exposure to environmentally relevant Cd levels causes persistent immunomodulatory effects in murine offspring. Thymocyte phenotype analysis determined that these effects are cell type-specific, whereas analysis of splenocyte phenotype demonstrates a cell type- and sex-specific effect. The cytokine profiles suggest an effect on peripheral Th1 cells in females and to a lesser degree in males. Others have noted different effects in the offspring of animals prenatally exposed to Cd, such as postaxial forelimb ectrodactyly (Scott et al., 2005), and delayed effects on renal function (Jacquillet et al., 2007) and sensorimotor developmental (Minetti and Reale, 2006), but this is the first report of such an effect on immune cell phenotype and function. The decrease in Th1 type cytokine production in females and the decreases in IFN-γ production and macrophage cell number in males may lead to increased susceptibility of the offspring to infections and tumor growth. These findings suggest that even very low exposure to Cd during gestation may result in long term detrimental effects on the immune system of the offspring, possibly resulting in cancer at adulthood, thus reinforcing that exposure to Cd during pregnancy should be limited.
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REFERENCES


CHAPTER 4: General Discussion I
GENERAL DISCUSSION I

The overall objective of this dissertation was to determine the effect of prenatal Cd exposure on the immune system. Specifically, our experiments were designed 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 in the thymus. In addition, longer term effects of prenatal Cd on the immune system were investigated. 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 of 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 overall hypothesis that prenatal Cd exposure dysregulates these signaling pathways in the thymus of the offspring, leading to changes in thymocyte phenotype, ultimately resulting in long term immunomodulatory effects.

It is well established that Cd exposure in adults results in immunotoxic effects (Descotes, 1992); however, studies on the effect of prenatal Cd exposure on the immune system of offspring are limited (Soukupova et al., 1991). In the U.S., more than a million newborns are exposed to cigarette smoke during gestation (DHHS, 2000). Cigarette smoke contains 1,000-3,000 ppb Cd. Several studies have indicated that smoking while pregnant increases the risk of certain types of childhood cancers, such as leukemias, lymphomas, and central nervous system tumors, in the prenatally exposed offspring (Filippini et al., 1994; Filippini et al., 2000; Schuz et al., 2001; Brooks et al., 2004). To an even greater extent, studies have linked maternal smoking with
respiratory disease, asthma and/or atopy in the offspring (Hu et al., 1997; Alati et al., 2006; Jaakkola et al., 2006; Pattenden et al., 2006; Raherison et al., 2007), thus it is critical to elucidate Cd’s role in these immunomodulatory effects. In this discussion, I will focus on the immunomodulatory effects of prenatal Cd exposure assessed at three developmental stages (PND0, 14, and 49) in murine offspring. In addition, I will describe a mechanism for the observed effects on PND0, and hypothesize possible mechanisms for the cell type-, sex- specific effects observed on PND14 and 49.

The focus of Chapter 2 of this dissertation addressed the effect of prenatal Cd exposure on thymocyte development in offspring at PND0. We hypothesized that prenatal Cd exposure alters thymocyte development due to a dysregulation in Shh and Wnt/β-catenin signaling. In order to understand how we arrived at this hypothesis, a brief review of the data leading up to this is required.

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 to the proliferation rate of thymocytes (Morselt et al., 1988). Several in vivo studies demonstrated that adult exposure to Cd is able to cause significant weight decrease or atrophy of the thymus in mice using a wide range of doses (Borgman et al., 1986; Mackova et al., 1996; Liu et al., 1999). To our knowledge, there have been no studies that have examined the effect of in vivo Cd exposure on thymocyte phenotype. 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; Pathak and Khandelwal, 2007), an increase in DN cells (Pathak and Khandelwal, 2007), and a decrease in the CD4+/CD8+ ratio (Dong et al., 2001; Pathak and Khandelwal, 2007).
The Hedgehog (Hh) and Wnt family proteins act as morphogens during thymocyte development. Shh signaling, 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. Analysis of Shh\(^{+/−}\) thymi showed that Shh is necessary for efficient proliferation of DN thymocytes (Shah et al., 2004). One study analyzing Shh’s role in the development of SP cells, showed that Shh signaling in developing thymocytes influences TCR repertoire selection and differentiation from DP to SP cells (Rowbotham et al., 2007). Specifically, in Shh\(^{−/−}\) fetal thymic organ cultures, the CD4\(^+\)/CD8\(^+\) ratio was increased. In addition to Hh proteins, the Wnt family of glycoproteins is involved in regulating thymocyte maturation (Staal et al., 2001). 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. Inhibition of the Wnt pathway results in reduced DN proliferation and differentiation, decreased DP survival (Verbeek et al., 1995; Okamura et al., 1998; Schilham et al., 1998), and decreased CD8\(^+\) generation (Mulroy et al., 2003).

Several studies have shown that prenatal Cd exposure dysregulates Shh signaling (Scott et al., 2005; Yu et al., 2006) and adult Cd exposure dyregulates Wnt/β-catenin signaling (Prozialeck et al., 2003; Thevenod et al., 2007; Thompson et al., 2008). Scott et al. (2005) demonstrated that administration of CdSO\(_4\) to C57Bl/6 mice at day 9.5 of gestation induces postaxial forelimb ectrodactyly in the offspring. This development malformation was due to Cd disrupting Shh signaling in the mouse limb.
bud. Although Shh signaling was decreased as measured by Shh-Light 2 cells, Shh transcription and translation was not affected, which led Scott et al. (2005) to propose that prenatal Cd exposure affects the processing of Shh in the cells in which it is made. Yu et al. (2006) quantified in vivo changes in Shh expression during early development in a green fluorescence protein transgenic zebrafish line following Cd exposure. The study showed that embryos affected by Cd-exposure demonstrated a down-regulation in Shh expression as determined by a decrease in the number of GFP-expressing cells measured by flow cytometry, and a decrease in expression of a downstream target of the Shh signaling pathway.

Prozialeck et al. (2003) demonstrated that Cd exposure in adult male Sprague-Dawley rats affects cadherin-dependent junctions in the proximal tubule epithelium, resulting in changes in localization of β-catenin. Thevenod et al. (2007) followed up this study with data proving that Cd induces nuclear translocation of β-catenin in proximal tubular cells, using a rat renal proximal tubule cell model. In addition, Thompson et al. (2008) demonstrated that Cd exposure induced nuclear translocation of β-catenin in peridermal and ectodermal cells when administered to post-gastrulation chick embryos.

Based on the aforementioned data that demonstrates an effect of adult Cd exposure on thymocyte development, a requirement of Shh and Wnt/β-catenin signaling for proper thymocyte development, and an effect of Cd exposure on Shh and Wnt/β-catenin signaling, we proposed to determine the effects of prenatal Cd exposure on thymocyte development, and to link those effects to a dysregulation in Shh and Wnt/β-catenin signaling. The data indicated that prenatal Cd exposure dysregulates two signaling pathways in the thymus, resulting in altered thymocyte development in murine
offspring at birth. To determine effects on thymocyte development, thymocyte phenotype was analyzed by flow cytometry. We showed that prenatal Cd exposure increased the DN4 subpopulation and the CD4⁺ cell population. Further, a significant increase in CD4⁺ cells and a trended decrease in CD8⁺ cells resulted in an approximate 10-fold increase in the CD4⁺/CD8⁺ ratio. In contrast to our findings, Dong et al. and Pathak and Khandelwal (2007) showed that in vitro Cd exposure decreased the CD4⁺/CD8⁺ ratio, however, the relevance of these studies to the interpretation of those reported herein is questionable because of the differences in experimental design such as in vitro vs. 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, which could have a significant impact on the phenotype. In our study, thymocytes were exposed to an indirect, environmentally relevant concentration of Cd. Although Cd was detected in pooled livers of the offspring, the level was close to the minimum level of detection, thus we believe that any possible direct Cd effect is minimal to nil. The increased CD4⁺/CD8⁺ ratio observed in our study could have several consequences in cell-mediated immunity and T-cell host response to infection in the offspring, including an increased chance of developing autoimmune disease and allergies, as well as an increased susceptibility to viruses and tumor cells.

To determine the mechanism for altered thymocyte development by prenatal Cd exposure, we analyzed several components of the Shh and Wnt/β-catenin pathways. Using the Gli: luciferase reporter cell line, Shh-Light 2, we observed an approximate 24% decrease in Shh signaling in the Shh-producing TECs, although this was not due
to a decrease in Shh or Gli1 protein levels. A decrease in mRNA level of Shh target gene *Wnt10b* in TECs further demonstrated a decrease in Shh signaling. Analysis of Shh target genes, *Ptc1* and *Gli1* in Shh-receiving thymocytes, showed that prenatal Cd exposure increased expression of Shh target genes, *Gli1* and *Ptc1*, in DN and CD8+ thymocytes, despite decreased Shh signaling in TECs. Based on our data and findings of others that suggest 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), 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, and this would contribute to the upregulation of *Gli1* transcription. Analysis of thymocyte phenotype showed a significant increase in the DN4 cell population as well as a trended increase in the DN3 cell population, thus, increased Shh signaling in the DN population may contribute to increased proliferation of these DN subpopulations. The role that Shh signaling plays in single positive cells has not been well defined, however, one study showed that Shh signaling in developing thymocytes influences TCR repertoire selection and differentiation from DP to SP cells (Rowbotham et al., 2007). Specifically, in Shh−/− fetal thymic organ cultures, the CD4+/CD8+ ratio was increased, therefore, the decreased Shh signal from TECs in the present study is likely contributing to the increased CD4+/CD8+ ratio observed in prenatal Cd-treated offspring. In addition, the increased transcription of *Ptc1* and *Gli1* in CD8+ and no change in these genes in CD4+ cells may also contribute to the significantly increased CD4+/CD8+ ratio observed in thymocyte phenotype analysis.
Our data also showed that Wnt/β-catenin signaling was decreased in thymocytes as determined by an increase in phospho-β-catenin in the cytoplasm and a decrease in ABC in the nucleus; however, this decrease was not due to a decrease in Wnt10b protein level in the TECs. Analysis of Wnt/β-catenin target genes, c-fos, c-jun, and c-myc, showed that mRNA levels were affected differentially among thymocyte populations. There was an overall upregulation of these genes in all of the thymocyte populations except the CD8+ population. Interestingly, thymocyte phenotype analysis showed that this was the only population that had a trended decrease following prenatal Cd exposure. Although c-fos, c-jun, 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. There are many proteins that are involved in β-catenin regulation, such as the destruction complex in the cytoplasm and inhibitory proteins in the nucleus, therefore one or a combination of those proteins may be affected by prenatal Cd exposure and contributing to our observed results.

Our data demonstrating the effect of prenatal Cd exposure on Shh and Wnt/β-catenin signaling implies that prenatal Cd exposure affects the processing and/or secretion of Shh and Wnt proteins in the thymic epithelium resulting in an attenuated signal in both pathways. In order for Shh and Wnt proteins to initiate proper signaling, they must go through a series of processing steps prior to secretion that involve multiple proteins. Any one of these steps could be a target of Cd, thus, further investigation should be conducted in order to elucidate this mechanism. Our results also imply that each thymocyte population responds uniquely to the decreased signals, as
demonstrated by varying target gene expression. One hypothesis for the altered signaling in thymocytes is that prenatal Cd is affecting Zn levels. Studies have shown that detrimental fetal effects have been attributed to Cd affecting Zn transportation from dam to fetus by the inhibition of transport proteins, thus decreasing Zn concentration in the fetal liver and placenta (Sowa and Steibert, 1985; Baranska-Gachowska et al., 1987). However, a study that used the same dose of Cd (10 ppm), the same strain of mouse (C57Bl/6), and the same day of analysis (PND0) as the study in Chapter 2, demonstrated that prenatal Cd exposure increased the hepatic and renal Zn concentration in the offspring, suggesting that the Zn deficiency may not be the only explanation for the effect of prenatal Cd (Ishitobi and Watanabe, 2005). To date, there have been no analyses of thymic Zn concentration following Cd exposure at any developmental stage. There is a requirement for Zn in the Shh (Hall et al., 1995; Koebernick and Pieler, 2002) and Wnt/β-catenin (Chen et al., 2009) pathways, therefore if prenatal Cd is disrupting Zn homeostasis in the thymus, it is likely that these pathways would be affected.

Another hypothesis for a mechanism that may contribute to Cd’s effect on Shh and Wnt/β-catenin signaling, involves activation of cellular protein kinases resulting in enhanced phosphorylation of transcription factors. Kinases activated following exposure of cells to Cd include protein kinase C (Beyersmann et al., 1994; Yu et al., 1997; Watkin et al., 2003), tyrosine kinase and casein kinase II (Saydam et al., 2002), stress activated protein kinase (Ding and Templeton, 2000), and the mitogen activated protein kinase family (LaRochelle et al., 2001). In the Wnt/β-catenin pathway, CKI and GSK3β are responsible for phosphorylating β-catenin, thus targeting it for ubiquitination
and degradation. Activation of these kinases would explain our findings of increased phospho-β-catenin in the cytoplasm and decreased active β-catenin in the nucleus of prenatal Cd-treated offspring. In the Shh pathway, protein kinase A (PKA) is responsible for phosphorylating Gli 2/3, thus targeting them for proteolytic processing to their smaller, repressor form. Activation of PKA would explain our finding of decreased transcription of Shh target gene, Wnt10b, in the thymus of prenatal-Cd treated offspring. Due to the combined complexity of each of the pathways and the reported effects of Cd on specific components of the pathways, a more global analysis of gene expression in each of the thymocyte populations should be undertaken so that the effect of prenatal Cd exposure on the Shh and Wnt/β-catenin pathways can be thoroughly elucidated.

The focus of Chapter 3 of this dissertation addressed the effect of prenatal Cd exposure on postnatal immune cell development and function. Due to organogenesis of the immune system occurring mostly at the prenatal, and to a lesser extent, at the early postnatal stage, the perinatal period is not only more sensitive to deleterious effects of immunotoxicants, but alterations in the immune system can result in persistent effects (Holladay and Smialowicz, 2000), therefore, we hypothesized that the effects on thymocyte development observed on PND0 would lead to continued effects on thymocyte and splenocyte development, as well as immune cell function at later developmental stages (PND14 and PND49). The data indicated that even a very low level of exposure to Cd during gestation can result in long term detrimental effects on the immune system of the offspring, and these effects are to some extent sex-specific. Prenatal Cd exposure increased the number of double negative (DN; CD4^-CD8^-) thymocytes, specifically the DN1 subpopulation (CD44^+CD25^-), at PND14 and 49. This
finding implies that prenatal Cd exposure has a continued effect on the thymus of exposed offspring. Due to the low transplacental transfer of Cd (Piasek et al., 2001), it is unlikely that we are observing a continued direct effect of Cd, thus we consider the effect to be immunoteratogenic. Analysis of splenocyte phenotype showed that prenatal Cd exposure decreased the number of macrophages, albeit only in males at PND14 and 49. This finding suggests that prenatal Cd exposure exerts a cell-type-, sex-specific effect in the spleen of exposed offspring. Moreover, this is a persistent effect. In vivo studies by others indicate that chronic Cd exposure alters the redox balance in adult male mice, inducing changes in lipid metabolism in macrophages, ultimately leading to apoptosis (Ramirez and Gimenez, 2002). In addition to being antigen presenting cells and phagocytes, macrophages can recognize tumor cells and induce cell death by releasing cytotoxic factors such as reactive oxygen and/or nitrogen intermediates, as well as cytokines; thus, the decrease in macrophage number in prenatal Cd-treated male offspring may increase susceptibility of males to tumor incidence. An analysis of macrophage function in male offspring should be conducted as well as additional studies to understand the mechanism of this cell type-, sex-specific effect.

Data on cytokine production on PND14 demonstrated that cytokine production (IL-2 and IL-4) was only decreased in females. Analysis at PND49 showed a dramatic Th1 type (IL-2 and IFN-γ) cytokine decrease by T cells from female offspring, as well as a decrease in IFN-γ secretion by T cells from male offspring. The IL-4 levels at PND14 reached comparable levels to control females by PND49, thus this cytokine may be affected in early development but returns to normal levels as the immune system
reaches maturity. In vitro studies have suggested that Th1 type cytokines (IL-2, IFN-\(\gamma\)) are depressed to a higher degree than Th2 type cytokines (IL-10, IL-4) following Cd treatment (Krocova et al., 2000; Hemdan et al., 2006; Pathak and Khandelwal, 2007), thus, our findings are to some extent, in support of the in vitro findings. Mouse studies evaluating sex differences following Cd exposure are limited; however, in vivo studies using adult female rats suggest that females may be at a greater risk than males for Cd-induced immunomodulation due to interactions between estrogen and Cd (Pillet et al., 2006). In addition, a finding that demonstrated Cd having a hierarchical effect on cytokine production in males may explain why males had decreased IFN-\(\gamma\) production but IL-2 was not affected. Pathak and Khandelwal (2007) demonstrated that IFN-\(\gamma\) is inhibited at a lower Cd concentration than IL-2 in adult male mice, so if female offspring in our study have a higher body burden or are more sensitive to prenatal Cd treatment, then a higher dose of Cd may be necessary to elicit a decrease in IL-2 in male offspring. Based on the data detailed in Chapters 2 and 3 of this dissertation, and data demonstrating addition of neutralizing anti-Shh antibody to anti-CD3/CD28 stimulated CD4\(^+\) T cell cultures reduced IL-2 and IFN-\(\gamma\) production, but not IL-10 production (Stewart et al., 2002), we hypothesize that the changes in splenic T cell cytokine production are linked to changes in morphogen signaling.

Since cytokines influence or control most immune responses, the decrease in Th1 cytokines caused by prenatal Cd exposure may increase the offspring’s susceptibility to bacterial and viral infections. Adult Cd exposure has also been shown to decrease host resistance in mice caused by viruses [murine coxsakie B3 virus (Ilback et al., 1995; Glynn et al., 1998) and the enterovirus, encephalomyocarditis (Gainer,
1977)], bacteria [E.coli (Cook et al., 1975), *Listeria monocytogenes* (Simonyte et al., 2003) and *Mycobacterium bovis* (Bozelka and Burkholder, 1979)], and parasites [*Hexamita muris* (Exon et al., 1975)]. The decrease in Th1 type cytokine production in females and the decreases in IFN-γ production and macrophage cell number in males, in our study, may lead to increased susceptibility of the offspring to infections and tumor growth, therefore; examination of host resistance in offspring exposed prenatally to Cd will be an important area of analysis.

In summary, data presented in this dissertation indicate that prenatal Cd exposure dysregulates two morphogen pathways required for proper thymocyte development, resulting in altered thymocyte phenotype in offspring on PND0. Further, modifications in thymocyte phenotype remained present in offspring at the adult stage, in addition to changes in spleen cell phenotype and function. These findings suggest that the defect caused by prenatal Cd exposure may be permanently encoded into the offspring and extends to secondary lymphoid organs. Therefore, even very low exposure to Cd during gestation may result in long term detrimental effects on the immune system of the offspring, possibly resulting in susceptibility to infections and disease at adulthood, thus reinforcing that exposure to Cd during pregnancy should be limited. The findings detailed in this dissertation are novel in that they are the first to: 1) demonstrate that prenatal exposure to Cd affects thymocyte development throughout post-natal development; 2) show that prenatal Cd dysregulates Wnt/β-catenin signaling in a mouse model, and 3) observe a sex-specific effect on immune cell phenotype and function following prenatal Cd exposure. The data presented herein provide a mechanism for prenatal Cd’s effect on thymocyte development in offspring observed on
PND0. This mechanism may be applicable to other detrimental effects of prenatal Cd exposure. In addition to the thymus, Hh and Wnt/β-catenin signaling is necessary for proper development of the kidney (Stark et al., 1994; Kang et al., 1997), bone (Gao et al., 2001; Gong et al., 2001; Loughlin et al., 2004), lung (Pepicelli et al., 1998; Tebar et al., 2001; Mucenski et al., 2003; Okubo and Hogan, 2004), and prostate (Podlasek et al., 1999; Truica et al., 2000; Lamm et al., 2002; Mulholland et al., 2002; Chesire and Isaacs, 2003; Freestone et al., 2003; Berman et al., 2004), all of which are target organs of Cd toxicity (Bernard, 2008). 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.
REFERENCES


APPENDIX: Subcellular localization of DCPA
Propanil, or 3, 4-dichloropropionanilide (DCPA), is a post-emergent herbicide used predominately for weed control in rice and wheat production (Dahchour et al., 1986). In 2001, an estimate by the Environmental Protection Agency (EPA) ranked DCPA as the 17th most used pesticide in the U.S., as a result of 50-70% of all rice grown in the U.S. being treated with the chemical. DCPA is used mostly in southern states along the Mississippi River and in some areas of California. The half-life of DCPA in the environment is 1-3 days in soil and 2-3 days in water (Wauchope et al., 1992). Due to the short half-life and limited use of the herbicide, the majority of exposed individuals are applicators and residents living near treated fields.

Rice and wheat crops have high levels of acylamidase activity, thus they are resistant to DCPA’s herbicidal effects due to their ability to enzymatically detoxify the chemical, while weeds lacking this enzyme are killed (Still and Kuzirian, 1967; Matsunaka, 1968). In humans and animals, the most common routes of exposure are inhalation, orbital, dermal, and to a lesser degree, oral. Following absorption, DCPA is metabolized by acylamidase into the major metabolite 3,4-dichloroaniline (DCA) (Williams, 1966). Other metabolites of DCPA include 2’hydroxypropanil and 6’hydroxypropanil (McMillan et al., 1990). DCA is then metabolized to 6-hydroxy-3, 4-dichloroaniline and N-hydroxy-3, 4-dichloroaniline. As a result of DCPA being readily metabolized, detection of DCPA in the blood of rats was detectable for approximately 48 hours following exposure (Izmerov, 1984).

Studies have demonstrated that DCPA is not mutagenic (McMillan et al., 1988), carcinogenic (EPA, 2003), or toxic to reproduction (Ambrose et al., 1972). Toxicity to
factory workers exposed to DCPA during its production and packaging has been reported (Morse et al., 1979). Symptoms of toxicity include blurred vision, small pupils, chloracne, muscle weakness, fatigue, and increased salivation (Morse et al., 1979). Animals studies have shown that manifestations of acute propanil toxicity include central nervous system depression (Singleton and Murphy, 1973), methemaglobinemia (Singleton and Murphy, 1973), and liver damage (Santillo et al., 1995).

DCPA has been extensively described as an immunotoxicant on both primary and secondary immune organs. Two studies have demonstrated DCPA’s myelotoxicity (Blyler et al., 1994; Malerba et al., 2002). Following exposure to 200 mg/kg DCPA, myeloid stem cells were reduced as analyzed using ex vivo colony-forming assays from bone marrow of acutely exposed C57Bl/6 mice (Blyler et al., 1994). Effects on early erythroid stem cells observed following in vitro exposure of human umbilical cord to DCPA supported the earlier report of myelotoxicity (Malerba et al., 2002). Erythroid colony-forming units (CFU-E) and erythroid burst-forming units (BFU-E) had 50% of their growth inhibited by 234 and 441 µM DCPA, respectively (Malerba et al., 2002). Several studies have indicated that the thymus is a target of DCPA’s toxicity (Barnett and Gandy, 1989; Cuff et al., 1996). In acutely exposed mice, thymic atrophy is observed approximately 2-4 days following exposure (Cuff et al., 1996). This atrophy has been attributed to increased levels of glucocorticoids (Cuff et al., 1996; de la Rosa et al., 2005). Following an intraperitoneal (i.p.) dose of 200 mg/kg, a reduction in thymic weight is present until 7 days following exposure (Barnett and Gandy, 1989), at which time the thymus has begun recovery from the atrophy (Cuff et al., 1996). The thymus reaches full recovery after 14 days following exposure (Cuff et al., 1996). In addition, in
vivo exposure to 200 mg/kg i.p. DCPA results in increased spleen weight (Barnett and Gandy, 1989).

Both innate and adaptive immunities are affected by DCPA exposure. The innate immune system is comprised of cell types including monocytes, macrophages, polymorphonuclear monocytes (PMN), and natural killer cells (NK), which serve as the first line of defense against invading pathogens. Studies have demonstrated that DCPA affects NK cells (Barnett et al., 1992; Pruett et al., 2000) and macrophages (Xie et al., 1997; Ustyugova et al., 2007) resulting in suppressed function of both cell types. DCPA also affects macrophage cytokine production. Thioglycollate-elicited macrophages from mice exposed in vivo by oral gavage to 400 mg/kg, or exposed in vitro to 33.3 \( \mu \text{M} \) DCPA had significantly decreased IL-6 and TNF-\(\alpha\) production in response to LPS stimulation (Xie et al., 1997). Further, in vitro phagocytosis of \textit{Listeria monocytogenes} bacteria by macrophages is decreased by DCPA exposure (Frost et al., 2001).

The adaptive immune system is comprised of B lymphocytes (B-cells) and T lymphocytes (T-cells). The primary function of B-cells is to produce and secrete antibodies. CD4\(^+\) T cells are responsible for activating and regulating cell-mediated immune responses and the humoral immune response, while CD8\(^+\) T cells destroy infected or transformed cells, thus they are called cytotoxic T lymphocytes (CTLs). Analysis of B cells in the periphery following DCPA exposure showed that although DCPA does not affect the number of B cells (Salazar et al., 2005), it does suppress some B cell functions (Barnett and Gandy, 1989; Barnett et al., 1992). In vivo exposure to 400 mg/kg DCPA suppresses splenic B cell proliferation following ex vivo stimulation with the B cell mitogen LPS (Barnett and Gandy, 1989). Analysis of antibody response
to T-dependent (TD) and T-independent type 2 (TI-2) model antigens showed that DCPA affects these responses to a greater degree than B cell proliferation (Barnett and Gandy, 1989; Barnett et al., 1992). DCPA has been demonstrated to decrease the TD antibody response to sheep red blood cells (SRBC) and the TI response to DNP-Ficoll (Barnett et al., 1992).

Studies have demonstrated that DCPA exposure results in an overall decrease in T cell number at several developmental stages (Zhao et al., 1995). Following exposure to 100 mg/kg DCPA, the number of all CD3+ (CD4+, CD8+, DP) and CD3- (DN) T cell populations in the thymus were decreased (Zhao et al., 1995), with the DP population being the most sensitive (Cuff et al., 1996). By day 7 after exposure, the percentage of cycling thymocyte populations returns to normal, suggesting that recovery from DCPA’s effect begins soon after exposure (Cuff et al., 1996). T cell function is differentially affected by DCPA. CTL function (Barnett et al., 1992) and cell mediated immune response to Listeria monocytogenes (Watson et al., 2000) were not affected by DCPA exposure. However, splenic T cell cytokine production of IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, and IL-6 were decreased in a dose-dependent manner following in vivo and in vitro exposure to DCPA and subsequent in vitro stimulation with concanavalin A (Zhao et al., 1998). DCPA was also shown to suppress IL-2 production in the murine T cell lymphoma line, EL-4, and the human T cell lymphoma line, Jurkat (Zhao et al., 1999; Brundage et al., 2004). DCPA exposure results in both a decrease in IL-2 transcription and an increase in degradation of IL-2 mRNA (Zhao et al., 1999). The Jurkat cell line has exhibited a greater sensitivity to DCPA than murine T cells. IL-2 mRNA levels were reduced by 77% 48 hours
following in vitro exposure to 5 µM DCPA (Brundage et al., 2004). In addition, 50 µM DCPA reduced the binding of IL-2 transcription factor, AP-1, to the DNA following one hour of exposure (Brundage et al., 2004). AP-1 is a heterodimer of two proteins, c-jun and c-fos. DCPA exposure decreases phosphorylation as well as protein levels of c-jun following one hour of exposure, which implies a potential mechanism for decreased IL-2 production (Brundage et al., 2004). Analysis of electron-spin resonance showed that DCPA affects a small component of the cell membrane, but the way in which this disruption affects IL-2 production has yet to be elucidated (Brundage et al., 2003). Many intracellular signaling events inhibited by DCPA exposure have one or more calcium (Ca²⁺) dependent steps. It was determined that DCPA exposure suppresses the normal elevated and sustained intracellular Ca²⁺ ([Ca²⁺]i) that follows internal store depletion in the human leukemic T cell line, Jurkat, and primary BALB/c mice T cells (Lewis et al., 2008). This inhibition of Ca²⁺ influx is consistent with inhibition of the Ca²⁺ release-activated Ca²⁺ (CRAC) channel. Recent findings suggest that DCPA inhibits CRAC channels by reducing the aggregation of the Ca²⁺ sensor, stromal interaction molecule 1 (STIM1) (Lewis et al, unpublished data). These data suggest that DCPA is a novel inhibitor of STIM-1 aggregation (Lewis et al, unpublished data).

Although the immunotoxic effects of DCPA have been extensively studied, the way in which DCPA modulates intracellular functions leading to these effects is less understood. In order to understand the mechanism of DCPA’s intracellular toxicity, it is necessary to determine whether the molecule actually transports into the cell. The following study was conducted with the intent to resolve this issue.
REFERENCES


Singleton, S. D., and Murphy, S. D. (1973). Propanil (3,4-dichloropropionanilide)-
induced methemoglobin formation in mice in relation to acylamidase activity.


Ustyugova, I. V., Frost, L. L., Van Dyke, K., Brundage, K. M., Schafer, R., and Barnett,

resistance to infection after acute exposure to 3,4-dichloropropionaniline. *Journal
of toxicology and environmental health* **60**, 391-406.

P. (1992). The SCS/ARS/CES pesticide properties database for environmental
decision-making. *Reviews of environmental contamination and toxicology* **123**, 1-
155.

the herbicide 3,4-dichloropropionanilide. *Toxicology and applied pharmacology* **9**, 459-500.

Xie, Y. C., Schafer, R., and Barnett, J. B. (1997). The immunomodulatory effects of the
herbicide propanil on murine macrophage interleukin-6 and tumor necrosis


Subcellular localization of the amide class herbicide 3,4-dichloropropionanilide (DCPA) in T cells and hepatocytes

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ABSTRACT

3,4-Dichloropropionanilide (DCPA), or propanil, a post-emergent herbicide used on rice and wheat crops in the United States, is immunotoxic in vivo and in vitro. Although it has been documented that DCPA exerts differential effects on specific immune cell types and is toxic to the liver, the subcellular localization from which DCPA modulates immune cells and hepatocytes has not been identified. In this study, Jurkat T cells were exposed to 100 µM DCPA for 1.5 hours after stimulation with anti-CD3/CD28. Following incubation, the cytosol, membrane-organelle, and nuclear/cytoskeletal fractions of the cells were isolated. In addition, hepatocytes from C57Bl/6 mice were exposed to 100 µM DCPA for 1.5 hours. Following incubation, the cytosol, light mitochondrial, and peroxisomal fractions of the cells were isolated. DCPA, when present, was removed from each cell fraction by liquid-liquid extraction. The extraction product was then analyzed for the presence of DCPA using liquid chromatography tandem mass spectrometry (LC-MS/MS). The cellular uptake of DCPA was monitored by detection of the molecular ion and product ion of DCPA. The analyses demonstrate that DCPA, a lipophilic compound, localizes primarily in the cytosol of T cells and hepatocytes. Low levels of DCPA were detected in the light mitochondrial fraction in the hepatocytes, while DCPA was absent in the membrane/organelle and nuclear/cytoskeletal fractions in T cells, and the peroxisome fraction in hepatocytes. These results indicate that DCPA is able to cross the plasma membrane and is accessible to intracellular immunomodulatory effectors.
Propanil (3,4-dichloropropionalide, DCPA) is an herbicide used extensively on rice and wheat crops in the United States. DCPA is in the acetanilide chemical family, and is a dichlorinated aromatic compound of low molecular weight. The major metabolite of DCPA is 3,4-dichloroaniline (DCA), which is mediated through an acylamidase-catalyzed hydrolysis of the parent compound (Singleton and Murphy, 1973; McMillan et al., 1990). Individuals involved in agriculture, in particular, are at risk for high-level exposure to DCPA. DCPA can be absorbed through the respiratory tract, the gastrointestinal tract, and the intact skin (Richards et al., 2001). In experimental animals, the half life of DCPA is rapid without respect to route of entry. Following a single intraperitoneal dose of DCPA in rats, the complete elimination from the body is within 48 to 72 hours (Izmerov, 1984). The pharmacokinetic profile of DCPA in humans is not available in the literature.

There are well-known immunotoxic effects on various compartments of the immune system following in vitro DCPA exposure, including T-helper lymphocytes (Cuff et al., 1996), B lymphocytes (de la Rosa et al., 2003), and macrophages (Frost et al., 2001). DCPA exposure decreases contact hypersensitivity responses, reduces proliferative responses to T-and B-cell mitogens, and impedes mixed lymphocyte reactions (Barnett and Gandy, 1989). Exposure to DCPA has also been found to induce splenomegaly and thymic atrophy in a dose-dependent manner in mice at 7 days postexposure (Barnett and Gandy, 1989). In addition to immunotoxicity, reports have demonstrated that DCPA is hepatotoxic to humans (De Silva and Bodinayake, 1997).
and rodents (Santillo et al., 1995). Following absorption by mammals, DCPA is quickly hydrolyzed, primarily to 3,4-DCA by the liver enzyme acylamidase (Williams, 1966). DCA is then metabolized to 6-hydroxy-3,4-dicloroaniline and N-hydroxy-3,4-dicloroaniline. Oxidation of DCPA occurs, most often, on the propionyl group of and at the 6-position on the phenyl ring (McMillan et al., 1990).

Although it has been documented that DCPA exerts differential effects on specific immune cell types and is hepatotoxic, to date, the subcellular localization from which DCPA modulates immune cells and hepatocytes has not been examined. The high lipophilicity of DCPA has led to the suggestion of a higher affinity of DCPA for membranes (Corsini et al., 2007). The aim of this study is to determine the localization of DCPA in T cells and hepatocytes following in vitro exposure. A combination of differential detergent fractionation (DDF) and centrifugation was used to thoroughly separate the cellular compartments. The product from a liquid-liquid extraction was analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Our results demonstrate that DCPA, a lipophilic compound (Finizio, 1997), localizes primarily in the cytosol of both T cells and hepatocytes. This study improves our understanding of the cellular location in which DCPA exerts its toxic effects.
MATERIALS AND METHODS

T cell treatment with DCPA

Experiments were performed using the human T cell leukemia cell line, Jurkat clone E6-1, obtained from the ATCC (American Tissue Culture Collection, Manassas, VA). Jurkat cells were maintained in complete Roswell Park Memorial Institute (RPMI) media (Mediatech Inc., Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (v/v) (FBS) (Hyclone Inc. Logan, UT), 100 units/ml penicillin (BioWhittaker), 100 µg/ml streptomycin (Bio Whittaker), 20 mM glutamine (BioWhittaker) and 50 µM 2-mercaptoethanol (Sigma, St. Louis, MO). The cultures were kept at 37°C in 5% CO₂. Cells in suspension were grown to obtain approximately 5 x 10⁷ cells. A 10 ml volume of cells was stimulated with anti-CD3 (10 µg/ml) and anti-CD28 (2 µg/ml) (BD PharMingen, San Diego, CA) and at the same time exposed to 100 µM DCPA or ethanol (vehicle control) for 1 hour (h) at 37°C, 5% CO₂. The cells were harvested and centrifuged at 1200 rpm for 8 minutes (min) at 4°C.

Protein Extraction by Differential Detergent Fractionation (DDF) and Centrifugation of T cells

DDF relies on detergents to sequentially extract proteins from eukaryotic cells. The cytosolic fraction was extracted by resuspending cells in 1 ml of 0.01% digitonin solution and incubated on ice for 30 min. The suspension was centrifuged at 14,000 x g for 2 min at 4°C. Digitonin interacts with cholesterol to form pores in the cell membrane.
and extract soluble proteins from the cytosol. The membrane/organelle fraction was extracted by resuspending the pellet from the digitonin extraction step in 0.6 ml 2% Triton-X 100 solution and incubated on ice for 1 h. The suspension was centrifuged at 14,000 x g for 10 min at 4°C. Triton X-100 solubilizes membrane and organelle proteins. The nuclear/cytoskeletal fraction was extracted by dissolving the pellet from the TX-100 extraction step in 1 ml 4% CHAPS solution for 1-2 h at room temperature (RT). The suspension was centrifuged for 10 min at 14,000 x g at RT to remove insoluble material. CHAPS results in the permeabilization of the nuclear envelope. The pellet was washed with Dulbecco’s phosphate buffered saline (DPBS) after each fraction removal to limit cross-contamination.

Western blot

Protein quantitation was determined using the 2D Quant Kit (Amersham Biosciences). A 30 µg aliquot of each fraction sample was boiled for 5 min to denature the proteins and electrophoresed through a 12% Tris polyacrylamide gel with a 4% stacking gel at 15 mAmps for 18 h. Proteins were transferred onto Hybond-P membranes (Amersham Pharmacia, Piscataway, NJ) at 200 V, 0.5 mA for 2 h. Blots were washed in TBS for 5 min at RT, blocked for 1 h in TBS + 0.1% Tween 20 (TBS/T) plus 5% dry milk at RT and then washed three times in TBS/T. Blots were incubated overnight at 4°C with primary antibodies specific for GAPDH, Oct-1, and Vdac/Porin (Santa Cruz Biotechnology, CA) in TBS/T plus 5% BSA. 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 a rabbit anti-goat IgG-HRP (Sigma-
Aldrich). 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).

**Mouse hepatocyte isolation**

Female C57Bl/6 mice at 8-10 weeks of age were obtained from Hilltop Lab Animals, Inc (Scottsdale, PA). Normal mouse hepatocytes were isolated using the method described by Muller *et al.* (Muller *et al.*, 1972). All animal methods were carried out utilizing an ACUC approved protocol. Briefly, mice were anesthetized utilizing IP injection of Pentabarbitol (100 mg/kg). The liver was exposed and perfused with Krebs Ringer with glucose (3.6 mg/ml) and 0.1 mM EGTA to remove the blood, while maintaining temperature with a heating lamp. Beldzyme 3 (Roche) was then perfused in slowly (50 ml of 0.02 mg/ml in Krebs Ringer plus glucose and CaCl₂ (1.37 mM). The liver was gently massaged following perfusion to assist in distribution of the enzyme solution. The liver was then carefully removed and minced in chilled perfusion buffer. Cells were dispersed with a large bore pipette and passed through a 70 µm filter to remove debris. Cells were washed 3 times by centrifugation (50 x g, 2 min, 4°C) with cold culture media (DMEM supplemented with 10% FBS and 100 units/ml penicillin, 100 µg/ml streptomycin, 20 mM glutamine and 50 µM 2-mercaptoethanol). Cells were cultured overnight on collagen coated dishes (37°C, 5% CO₂) prior to exposure to DCPA.
Peroxisome purification

Overnight cultures of isolated primary hepatocytes were incubated with 100 μM DCPA or ethanol for 1.5 h at 37°C, 5% CO₂ prior to preparation of peroxisomes. Cells were scraped from the dishes and pelleted (300 x g, 10 min, 4°C). The resulting cell pellets were resuspended in 3 ml of ice cold homogenization buffer (HM; 9% sucrose, 1mM EDTA, 10mM Tris, 5mM MOPS, 0.1% ethanol, pH 7.2 ). The resuspended cells were homogenized on ice using 50 strokes of a P/J homogenizer fitted with a Teflon pestle and attached to a variable speed hand drill. The homogenate was centrifuged (500 x g, 5 min, 4°C) and the supernatant was saved. The remaining cell pellet was re-homogenized and centrifuged. The resulting supernatant fractions were combined (crude homogenate fractions). The crude homogenates were centrifuged in corex tubes (6000 x g, 10 min, 4°C). The pellets were resuspended in 3 ml of ice cold HM (Pellet #1). The resulting supernatants (Supernatant #1) were centrifuged in corex tubes (20,000 x g, 15 min, 4°C). The resulting supernatant was reserved (Supernatant #2-Cytosol Fraction) and the pellet was resuspended in 3 ml of ice cold HM buffer (Light Mitochondrial Fraction). The resuspended pellet was then centrifuged over a cushion of 30% Optiprep (Sigma) using a Ti50.2 rotor (40,000 x g, 3.25 h, 4°C) The pellet was then resuspended in 600 μl of ice cold HM buffer (crude peroxisome fraction)

Mass spectrometric analysis of cell fractions

Volumes of all fractions were adjusted using PBS to achieve a protein concentration of 0.5 μg/μl. DCPA was extracted from cell fractions using a liquid-liquid extraction procedure as follows: 25 μl of each cell fraction was diluted to 500 μl with
deionized water. Then, 50 µl of the internal standard acetaminophen was added at a final concentration of 1.1 µg/ml. To the vortexed sample, 2 ml of ethyl acetate was added and shaken vigorously. This served to partition the DCPA and acetaminophen into the organic phase. The samples were centrifuged at 3000 rpm for 5 min at RT to ensure separation of layers before keeping the ethyl acetate extract. The organic portions were dried under a stream of nitrogen and reconstituted with 100 µl of deionized water. A 5-point standard curve of a DCPA concentration range of 5-500 ng/ml was used to estimate the quantity of DCPA in cell fractions.

LC-MS/MS was used to determine DCPA localization in cells. High performance liquid chromatography (HPLC) separation was achieved using a reversed-phase C8 (50 mm x 4.6 mm) column with a flow rate of 200 µl/min and a gradient as follows: 80% mobile phase A (0.05% formic acid, aq) from 0-1.5 min; increased from 20-60% mobile phase B (0.05% Formic acid in acetonitrile) from 1.5-3.5 min; 60% B held from 3.5-7 min; column re-equilibrated from 60-20% B from 7-9 min. Tandem mass spectrometry operated in the positive ion mode with sheath gas (nitrogen) flow rate: 80 arbitrary (arb.) units (with 20 arb. units auxiliary gas flow rate), spray voltage 5.2 kV, capillary voltage 9 V, and heated capillary temperature 300°C. Acetaminophen eluted at approximately 2 min. From 0-4 min, MS/MS was performed on m/z 152 (acetaminophen) and the selected ion monitored (SRM) range was set for m/z 110 (fragment of acetaminophen). From 4-10 min, MS/MS was performed on m/z 218 ± 4 amu (to encompass all ionized forms of DCPA of m/z 216, 218, and 220). A SRM was set up with a range of m/z 162 ± 4 amu (to encompass all DCPA MS/MS fragments of m/z 160, 162, and 164, respectively).
Results and Discussion

Analysis of fractionated T cells following stimulation and treatment with 100 µM DCPA for 1.5 h demonstrated that DCPA localizes in the cytosol (Figure 1A) at a concentration of 26.26±2.02 ng/ml (Table 1). DCPA was not detected in the membrane/organelle fraction (Figure 1B) or in the nuclear/cytoskeletal fraction (Figure 1C). Therefore, DCPA, a lipophilic molecule, passes through the plasma membrane and resides in the cytosol of T cells following 1.5 h of exposure.

Analysis of fractionated hepatocytes following treatment with DCPA for 1.5 h demonstrated that the molecule localizes mostly in the cytosol (Figure 2A) at a concentration of 120.24±5.78 ng/ml (Table 1). DCPA was also detected in the light mitochondrial fraction (Figure 2B), but the level was minimal relative to the level detected in the cytosol (Table 1). There was no DCPA detected in the peroxisomal fraction of hepatocytes (Figure 2C). Therefore, DCPA localizes predominately in the cytosol of hepatocytes following 1.5 h of exposure.

The greater DCPA concentration observed in the hepatocyte cytosol (120.24±5.78 ng/ml) compared to that in the T cell cytosol (26.26±2.02 ng/ml) is likely attributed to differences in cell size and cell composition. Hepatocytes are among the largest of cells, with an average diameter of 18-22 µm. In addition, they display an eosinophilic cytosplasm which contains numerous mitochondria. The high density of mitochondria present in hepatocytes may contribute to identification of DCPA in those organelles in hepatocytes and not T cells. T cells have an average diameter of 7-12 µm with the nucleus being the major constituent of the cell, thus there is little eosinophilic
cytoplasm. The finding that DCPA is only detected in the cytosol of T cells coupled with the size of this constituent, suggests that DCPA’s mechanism of action has specificity.

We hypothesized that DCPA exerts its effects on immune cells and hepatocytes by interfering with cell activation and cell signaling processes at the cell membrane, due to DCPA being a highly hydrophilic, lipophilic compound. Our finding that DCPA is not in the membrane does not eliminate the possibility that DCPA is affecting membrane proteins or channels as it migrates into the cytosol. An earlier finding in our lab demonstrated that DCPA exposure does not disrupt the motional properties of lipid hydrocarbon chains in the bilayer, but does alter distribution of lipids in distinct motional environments in the membrane (Brundage et al., 2003). The present study provides an explanation for the limited effect observed in hydrocarbon chain mobility. The finding that DCPA is able to move through the membrane of T cells and hepatocytes and localize in the cytosol will aid in the understanding of DCPA’s mechanism of toxicity to cellular functions.
Figure 1

A

B

C

Intensity (x10^5)

Time (min)

DCPA

239
Figure 1. Chromatograms of DCPA localization in T cells. Jurkat T cells were stimulated with anti-CD3/CD28 and exposed to 100µM DCPA for 1.5 hours. Cells were fractionated using DDF and centrifugation. LC-MS/MS was used to determine DCPA location in cell fractions. Acetaminophen was used as an internal standard. Protein concentration of each fraction was 0.5 µg/µl. (A) Cytosol fraction, (B) Membrane/Organelle fraction, (C) Nuclear/Cytoskeleton fraction. Representative of 3 separate experiments.
Figure 2

A

B

C

Intensity ($\times 10^6$)

Time (min)

DCPA

DCPA

DCPA
**Figure 2. Chromatograms of DCPA localization in hepatocytes.** Primary mouse hepatocytes were exposed to 100 µM DCPA for 1.5 hours. Cells were fractionated using homogenization and centrifugation. LC-MS/MS was used to determine DCPA location in cell fractions. Acetaminophen was used as an internal standard. Protein concentration of each fraction was 0.5 µg/µl. (A) Cytosol fraction, (B) Light Mitochondrial fraction, (C) Peroxisome fraction. Representative of 3 separate experiments.
Table 1. DCPA concentration in T cell and hepatocyte fractions.

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<th>DCPA concentration (ng/ml)</th>
<th>Hepatocytes</th>
<th>DCPA concentration (ng/ml)</th>
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<td>Cytosol</td>
<td>120.24±5.78</td>
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<td>Membrane/organelle</td>
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Each value is mean ± SEM, n=3
REFERENCES


factor nuclear factor-kappab in the macrophage cell line ic-21. Toxicology and applied pharmacology 172, 186-193.


GENERAL DISCUSSION II

The aim of the study described in the Appendix of this dissertation was to determine the subcellular localization of the herbicide DCPA. Due to the immunotoxic effects of DCPA on T cells (Barnett et al., 1992; Zhao et al., 1995; Cuff et al., 1996; Zhao et al., 1998; Zhao et al., 1999; Brundage et al., 2004; Lewis et al., 2008), the localization of DCPA in Jurkat T cells was determined. Hepatocytes were also analyzed in the study due to the liver being the major site for metabolism of DCPA and because of reports demonstrating that DCPA is hepatotoxic to humans (De Silva and Bodinayake, 1997) and rodents (Santillo et al., 1995). We hypothesized that due to DCPA’s lipophilic and hydrophobic nature, it would localize in the cell membrane. Analysis of the cytosol, membrane-organelle, and nuclear fractions of T cells demonstrated that DCPA does not reside in the membrane, but rather accumulates in the cytosol. Similarly, analysis of the cytosol, light mitochondrial, and peroxisomal fractions of hepatocytes demonstrated that DCPA accumulates mostly in the cytosol, while minimal levels were detected in the light mitochondrial fraction. The results suggest that cell size and composition affects DCPA accumulation and, to some degree, its localization. Knowing the subcellular localization of DCPA in T cells and hepatocytes will aid in the understanding of DCPA’s mechanism of toxicity to cellular functions.
REFERENCES


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1996-2000: Mount de Chantal Academy, Wheeling, WV
2001-2005: Wheeling Jesuit University, B.S. in Biology
2005-2009: West Virginia University, School of Medicine, Immunology and Microbial Pathogenesis, PhD

Employment History
2003-2004: Organic Chemistry Lab, Wheeling Jesuit University, Teaching Assistant
2003-2005: Academic Resource Center, Wheeling Jesuit University, Writing Tutor
2004-2005: Academic Resource Center, Wheeling Jesuit University, Biology Tutor
June-August 2004: Mary Babb Randolph Cancer Center, West Virginia University, Intern
2004-2005: Molecular Biology, Wheeling Jesuit University, Lab technician
2005-Present: Graduate Research Assistant, West Virginia University
August-December 2007, August-December 2008: Medical Microbiology Lab, Teaching Assistant
Sept 1, 2009-Present: National Cancer Institute, Frederick, MD, Post doc

Awards
May 2005: Thacker Award for Performance in Biology, Wheeling Jesuit University
May 2005: Haig Award for Scientific Research, Wheeling Jesuit University
April 2008: Keynote Speaker, Research and Scholarship Day, Wheeling Jesuit University

Presentations
2004-2005: AFAP-110 as a potential binding partner of the p85α subunit of phosphatidylinositol kinase, WV-INBRE Symposium, West Virginia University; Research Day at the Capitol, Charleston, WV; Research and Scholarship Symposium, Wheeling Jesuit University
December 2007: The effect of prenatal cadmium exposure on thymocyte development, Department of Microbiology, Immunology, and Cell Biology, West Virginia University
March 2008: Subcellular localization of the amide class herbicide 3,4-dichloropropionanilide (DCPA) in T cells, Society of Toxicology Conference, Seattle, Washington
October 2008: Immunotoxic effect of prenatal cadmium exposure on murine offspring, West Virginia COBRE/INBRE Conference, sponsored by NIH's National Center for Research Resources
March 2009: Immunotoxic effect of prenatal cadmium exposure on murine offspring, Society of Toxicology Conference, Baltimore, Maryland
Publications


Professional Memberships

2007-Present: American Association of Immunologists (AAI)
2008-Present: Society of Toxicology (SOT)
2008-Present: American Medical Writers Association (AMWA)

References

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