Chapter 5: Hepatomegaly and diminished hematopoiesis in C57Bl/6 fetal mice after exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an agent that targets progenitor immune cells: relationship to altered c-jun, PKCα, and p53 expression.

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5.1 Abstract: TCDD has known myelotoxic and lymphotoxic effects when administered to mice. Prior studies document diminished numbers and impaired function of T-cell progenitors in the fetal mouse liver after gestational exposure to TCDD, however light microscopic changes in liver morphology have not been described. Treatment of pregnant C57Bl/6 mice with 10 μg/kg TCDD on gestation days (gd)14 and 16 led to increases in fetal liver-to-body weight ratios and absolute increases in liver weights on gd18 relative to vehicle-treated controls. Histologically, cytomegaly and increased cytoplasmic basophilia were seen within hepatocytes after TCDD treatment. TCDD increased ratios of hepatocytes to hematopoietic cells, however megakaryocyte numbers were unaffected Real time quantitative PCR demonstrated that TCDD increased c-jun mRNA gene expression levels in whole fetal liver. Decreases in p53 mRNA without alteration in Bcl-2 expression suggest a pro-proliferative and anti-apoptotic status of the cells after TCDD exposure, while noted decreases in expression of PKCα may indicate decreases in phosphorylation of substrates required for normal cell cycle progression. These results are reminiscent of known myelotoxic effects of TCDD in adult mice, and, further, suggest metabolic capacity of late gestation fetal liver for this compound.
Keywords: dioxin, developmental, hematopoiesis, liver, murine

5.2 Introduction

One of the most immunotoxic of the halogenated aromatic hydrocarbons, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is a ubiquitous environmental contaminant. TCDD is a by-product of chemical synthesis, manufacturing, and combustion. Human environmental, occupational, and accidental exposures to TCDD occur, and are of concern as this agent is a known carcinogen, teratogen, and endocrine disrupter (reviewed by Birnbaum and Tuomisto, 2000; reviewed by Mann, 1997). TCDD crosses the murine placenta, and can be found in fetal tissues in readily measured quantities (Nau and Bass, 1981; Nau et al, 1986).

Gestational administration of TCDD results in fetal thymic atrophy, an effect that appears to have multiple shared etiologies. Increased thymocyte apoptosis (Kamath et al, 1997, 1999; Besteman et al, 2005), alteration of thymocyte maturation (Blaylock et al, 1992) and antiproliferative activity of TCDD (Comment et al, 1992) are among mechanisms contributing to thymic atrophy. Other studies have shown that the prothymocyte is an exquisitely sensitive target of immunotoxicants (Fine et al, 1990a; Fine et al, 1990b; Silverstone et al, 1992) and that impact at this level may make a substantial contribution to thymic atrophy described after TCDD administration.

In the murine fetus, the liver is the primary hematopoietic organ, and therefore the source of thymocyte progenitor cells. Between gestational days 10 and 12 the pluripotent hematopoietic stem cells migrate from the aorta-gonodo-mesonephros region to the liver. For the first few days of residence within the liver, these pluripotential stem cells expand via self-renewal, contributing to the increasing the size of the hematopoietic population.

Differentiation of the hematopoietic cells occurs in parallel to this expansion process until the bone marrow assumes these functions after birth (Douagi et al, 2002).

Hackney et al (2002) demonstrated that the fetal liver stromal population both supports and contributes to the regulation of hematopoiesis. Supportive stem cell lines derived from fetal livers successfully maintained hematopoietic stem cells, permitting their self renewal without differentiation. Using a fetal liver culture and fetal hematopoietic cells, results of Kinoshita et al (2001) also suggested that liver cells indirectly influence lymphopoiesis via the production of cytokines and stimulating factors. Most recently, using hepatocyte cell lines in an *in vitro* system, purified hematopoietic stem cells (Sca-$1^{+}Lin^{-}$) differentiated into natural killer (NK) cells (Bordoni et al, 2004). These results indicate that the hepatocytes themselves may play a direct role in NK cell development and, further, that alterations of the hepatocytes may impact lymphopoiesis. Taken together, these studies suggest that the fetal hepatic environment may play a vital role in the developing hematopoietic compartment *in vivo*.

Histopathologic evaluation after gestational administration of TCDD was performed in this experiment in order to characterize the effect this immunotoxic compound has on the fetal liver, in both the hematopoietic and hepatocytic compartments. As fetal tissues are often concurrently undergoing proliferation and apoptosis, a small panel of well characterized genes (Bcl-2, p53, pkCα, and c-jun) was used to assess the potential influence TCDD may have on these two processes.

5.3 Materials and Methods

Animal Model. Eight-week-old C57Bl/6 timed pregnant female mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) on the morning of gestational day 14 (gd

14). These mice were arbitrarily distributed into control and treatment groups. Mice were housed from gd 14-16 in groups of 3-5 per cage and provided with Harlan Teklad Global 2018 18% Protein Rodent Diet (Harlan) and distilled water *ad libitum*. A constant temperature of 21 ± 2 ºC was maintained, and a 14.5/9.5 hour light/dark cycle used in the facility. After the final vehicle or TCDD administration, the mice were housed individually until time of sacrifice on gd 18. These and all other procedures for these mice were reviewed and approved by the Virginia Tech Animal Care and Use Committee prior to initiation of the experiments.

Chemical Exposure. TCDD (AccuStandard, Inc., New Haven, CT) was dissolved in corn oil (Sigma Aldrich) to a concentration of 1.8 μg/mL and administered in a volume of 120 – 200 μL (10 μg/kg exposure) by oral gavage on gd days 14 and 16. Control mice were administered comparable volumes of corn oil vehicleby oral gavage on the same dates.

Tissue Collection. On gd 18, mice were sacrificed by cervical dislocation and fetal mice collected. Total fetal weight per litter as well as total fetal number per litter were recorded. Fetal mice were placed on ice until motionless. For organ weight and microscopic studies, fetal livers were then removed and placed in pre-weighed culture dishes containing 2 mL RPMI (Sigma Aldrich, St. Louis, MO). Dishes were immediately re-weighed (Mettler Toledo PB303, Carlton Scale, Roanoke, VA). Two livers from each litter were then arbitrarily removed from culture dishes and submersed in 10% neutral buffered formalin for a minimum of 24 hours for histologic study.

For the subsequent gene expression experiments, fetal livers were removed from the animals and immediately placed, by individual litter, in RNALater solution (Ambion Inc., Austin, TX) and frozen at -20C until RNA isolation could be performed.

Histopathology. Litters from five control and five TCDD treated dams were used for light microscopic evaluation. Two fixed fetal livers from each litter were embedded in paraffin, sectioned at 6 microns, and stained with hematoxalin and eosin. Stained sections were assessed for hepatocellular and hemopoietic alterations at 100x and 500x magnification. A minimum of four separate fields of 1000x magnification in the central parenchyma excluding portal regions but within the borders of the liver were used to determine cell ratios. A population of small cells with deeply staining nuclei, scant basophilic cytoplasm, and a compact chromatin pattern was identified. These mononuclear cells were interpreted as either hematopoietic stem cells or small lymphocytes. Counts of hematopoietic cells vs. hepatocytes were made in the intralobular areas between the portal veins. Cells were also assessed for indications of morphologic changes associated with apoptosis such as pyknotic nuclei.

Those sections considered of poor quality due to fractures in the tissue, folds of tissue, autolysis, or inadequate staining were excluded when evaluating architecture and other indices.

cDNA synthesis: Four control and four TCDD treated dams were used for gene expression experiments. A minimum of two livers from each litter were used for RNA isolation. RNA was extracted from the fetal livers using Trizol (Gibco, Rockville, MD) according to manufacturer protocol, and then dissolved in DNAse- and RNAse-free distilled water (Gibco, Rockville, MD). Reverse transcription of the RNA was performed

using 2 μg of total RNA and the Promega Reverse Transcription System (Madison, WI) according to the manufacturer protocol.

Quantitative PCR: A100ng RNA-equivalent was used for each reaction in quantitative PCR. TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and pre-developed primer/probes (Qiagen, Valencia, CA) specific to c-jun, Bcl-2, p53, and PKCα were used according to manufacturer protocol with the ABI 7700 (Applied Biosystems, Foster City, CA) to quantitate the expression of genes relative to controls. 18S rRNA (Applied Biosystems, Foster City, CA) was used as the internal reference.

Statistics

The two-tailed Student's *t*-test was applied to weight data, data involving litter size, and histopathologic findings that were numerically evaluated. Data means described as different in this report were significantly different at $p \leq 0.05$. Some observations, such as alterations of architecture or cellular morphology, were descriptive.

Quantitative PCR results were analyzed by the Virginia Tech Statistical Consulting Center using the Dunnett's t-test to compare the treatment group to the control group. The comparative threshold cycle (CT) method described by Livak and Schmittgen (2001) was employed for evaluating gene expression results, with target expression first normalized to 18s rRNA within each sample (ΔCT), then compared between TCDD- and vehicle-treated groups (ΔΔCT).

5.4 Results

Weights No differences were found in fetal number per litter between the treated and control groups (Table 5.1). No differences in pooled fetal weights or individual fetal weights were noted after TCDD treatment (Table 5.1). The pooled fetal liver and

individual fetal liver weights were increased after TCDD exposure. Ratios of pooled liver weights to pooled fetal weights, expressed in percentages, were increased in the treatment group relative to the controls.

Morphology

Description of normal, late gestation murine fetal liver was not found in the literature. Grossly, increased size was the only difference between TCDD treated fetal livers and controls. Using light microscopy, the corn oil control livers had clearly visible central veins but portal triads were inconspicuous as were sinusoidal spaces and individual plates of hepatocytes (Figure 5.1A). Hepatocytes contained discrete, large intracytoplasmic vacuoles displacing their oval vesicular nuclei to the cell margins. A small amount of an amorphous eosinophilic intracytoplasmic material (presumed glycogen) was noted. Dense, multifocal areas of extramedullary hematopoiesis were represented by occasional megakaryocytes and moderate numbers of hematopoietic stem cells, erythroid and myeloid progenitor cells, as well as more differentiated cells distributed diffusely throughout the parenchyma (Figure 5.2A). Low numbers of predominantly mature red blood cells along with occasional metarubricytes were present in the poorly visualized hepatic sinusoidal spaces and within the central vein lumen. Rare bile ducts in triad regions, lined by plump cuboidal epithelial cells having oval centrally located nuclei and prominent single nucleoli, were also identified.

TCDD treated fetal livers showed a decreased hepatocellular vacuolation and a concomitant increase in cytoplasmic basophilia (Figures 5.1B, 5.2B). Hepatocytes had more variable features than those in the control group. Nucleoli were more prominent and hepatocytes demonstrated mild to moderate cytomegaly. Megakaryocytes remained

constant regardless of treatment, with no significant change in number or appearance. The nests of small mononuclear cells present were decreased in overall number as well as numbers of cells within each hematopoietic cluster. Total small mononuclear cell counts were significantly decreased relative to controls and relative to the hepatocyte population present (Table 5.2).

Quantitative PCR Analysis: Fetal livers treated with TCDD demonstrated increases in the expression of c-jun and decreases in the expression of p53 relative to fetal livers from corn oil-treated pregnant dams (Figure 5.3). TCDD treatment also resulted in a modest decrease in PKCα expression relative to controls.

5.5 Discussion

The development of the immune system begins early in embryonic life following a series of events required for the normal pattern of ontogenesis and function. These events include the initial hematopoietic cell development, the migration of these cells to appropriate hematopoietic organs, the intimate intercellular interactions involving select microenvironmental influences, specific steps for cellular differentiation, and final maturation allowing for distinctly defined functional properties of each cell (Holladay and Smialowicz, 2000) Previous reports indicate that the developing immune system has a greater sensitivity to immunotoxic agents than the adult, and that gestational/perinatal exposure may result in more dramatic or more persistent effects (Holladay and Luster, 1996; Holladay and Smialowicz, 2000; Luebke et al, 2006).

Prior studies have examined the myelotoxicity of a number of chemicals, including TCDD (reviewed by Boorman et al, 1982). TCDD exposure led to a diminution of total bone marrow cellularity as well as pluripotent stem cells and granulocyte-macrophage

progenitors. Sakai et al (2003) reported that treatment with TCDD diminished the long term reconstituting activity of hematopoietic stem cells, even though the numbers of these $CD34^-$, c-kit^+, Sca-1^+ lineage negative cells increased after a single oral administration of TCDD in adult C57Bl/6 mice. In another study, TCDD caused a decrease terminal deoxynucleotidyl transferase-synthesizing cells (a progenitor T cell marker) in the bone marrow as well as the thymus in adult BALB/cJ mice (Silverstone et al, 1992).

TCDD has long been described as an hepatotoxic agent in the rodent model (reviewed by Birnbaum and Tuomisto 2000, and Mann, 1997). Documentation of enzyme induction, fatty infiltration, cytomegaly, cytotoxicity, necrosis, reparative hyperplasia, and proliferative response have been reported after TCDD administration. Previous gestational exposure of Sprague-Dawley rats on gd 17 with a 2.5 ug/kg intraperitoneal injection of TCDD resulted in extensive cellular necrosis in the fetal livers, giving the livers a mottled appearance grossly, while electron microscopy revealed enlarged and swollen mitochondria, increased rough endoplasmic reticulum, and increased glycogen deposits (Berry et al, 1976). Increases in enzyme activity in the TCDD treated rat fetal livers were also noted. Enzyme induction by TCDD has also been documented in C57Bl/6 adult mice, and included increases in gene expression of CYP1A1, CYP1A2, 7-ethoxyresorufin-O-deethylase and 7-methoxyresorufin-O-deethylase (Vogel et al, 1997). Increases in liver weight, both absolute and relative to body weight, were noted after administration of a single dose of TCDD in adult C57Bl/6 mice (Birnbaum et al, 1990). Liver weight to body weight ratios were also increased in adult Sprague-Dawley rats

treated with TCDD, in conjunction with periportal hepatocyte proliferation (Fox et al, 1993).

Grossly the TCDD fetal livers in the present experiment did not differ from controls except for their relative sizes. The microscopic findings of increased cytoplasmic basophilia and mild to moderate cytomegaly are consistent with previous descriptions of reparative hyperplasia, enzyme induction, and glycogen deposition seen after TCDD treatment. The increased liver to body weight ratios in TCDD treated feti seemed to be most compatible with the prior reports of hepatic hyperplasia and cytomegaly. No indications of necrosis were found in either control or treated fetal livers in the present study.

The most noteworthy changes in hematopoiesis detected histologically were the relative diminution of small mononuclear cells with dense nuclear chromatin and scant cytoplasm (compatible with hematopoietic stem cells or small lymphocytes) along with an overall decrease in hematopoiesis. Interestingly, thrombopoiesis appeared unaffected by treatment. The architectural organization of the liver was affected by the overall diminution of hematopoiesis after treatment with TCDD, as the nests of stem and progenitor cells were of lower cellularity and were more widely scattered.

Preliminary gene expression analysis was conducted to suggest molecular pathways through which TCDD may be impacting the fetal liver. Histology indicated a proliferative activity in the hepatocytes and an alteration in hematopoiesis, therefore genes that are known to impact those two activities in these cell populations were chosen for evaluation.

c-jun is a proto-oncogene and transcription factor (Hartl et al, 2003). Previous studies using a c-jun null mouse model have shown that c-jun is essential for normal murine embryonic survival and development (Hilberg et al, 1993; Johnson et al, 1993). Further, it is essential for normal hepatic development (Behrens et al, 2002; Eferl et al, 1999) as well as having a role in hematopoietic differentiation, proliferation, and apoptosis (Mouthon et al, 1992; Liebermann et al, 1998; Szabo et al, 1991; Lord et al, 1993; Shimizu et al, 1996). Increased expression of c-jun in the current experiments after TCDD administration suggests that c-jun may play a role in the cytomegaly and apparent hyperplasia of the hepatocyte population. No alterations were noted in the morphology of individual hematopoietic cells to indicate that the increase in c-jun correlated with alterations in differentiation. While apoptosis of the hematopoietic cells was not appreciated histopathologically, c-jun has been shown to be a positive modulator of apoptosis in hematopoietic cells (Liebermann et al, 1998), and an increase in apoptosis may contribute to the hematopoietic depletion of the TCDD treated fetal liver.

PKCα has been implicated as a necessary modulator of the aryl hydrocarbon receptor (AhR), and therefore has a role in the receptor mediated signal transduction after TCDD administration (Carrier et al, 1992; Chen and Tukey, 1996; Long et al, 1998). PKCα signaling has also played a role in thymocyte lineage commitment (Ohoka et al, 1997), as well as the differentiation of hematopoietic progenitor cells (Rossi et al, 1996; Myklebust et al, 2002; Darley et al, 2002; Pierce et al, 1998). Suppression of the PKCα isoform has been associated with increased apoptosis in hepatic cell lines (Hsieh et al, 2003). In this experiment, an overall decrease in PKCα can not be attributed solely to either hematopoietic or hepatocellular expression but may, in fact, be the result of changes in

expression of both of these cell populations. These results suggest a nonspecific alteration in the phosphorylation status of different cellular substrates that may have impacted a variety of cellular functions.

Bcl-2 has long been characterized as an anti-apoptotic gene, however its role in hematopoiesis has recently been described (Orelio et al, 2004). Overexpression of Bcl-2 resulted in increased hematopoietic stem cell activity suggesting that apoptosis may play a part in normal hematopoietic homeostasis. Bcl-2 affects the longevity of dendritic cell populations as well (Nopora and Brocker, 2002). Another study indicated that Bcl-2 played a key role in the development of lymphoid cells, however it was not essential for nonlymphoid hematopoiesis (Matsuzaki et al,1997). Its expression in the production of thymocytes is variable depending on their stage of development (Veis et al, 1993). In adult mouse liver, Bcl-2 increases were noted in lesions after chronic treatment with TCDD, and primarily in the preneoplastic foci (Christensen et al, 1999). However, in the present experiments no significant alteration of Bcl-2 expression occurred after TCDD administration. This may be consistent with the gender specific response of the Sprague-Dawley rat after a single gestational exposure (Chang et al, 2005). Male rat offspring demonstrated no change in Bcl-2 protein expression in the liver on day 0 of neonatal life, while females demonstrated a downregulation in Bcl-expression on that same day. In another study, TCDD did not significantly alter the expression of Bcl-2 in hematopoietic cells (Puebla-Osorio, 2004). It is possible that the expression of Bcl-2 in the present experiment is a net effect of its up- or down-regulation in the two predominant cell populations. Separation of hematopoietic and hepatocytic cell lines

would afford a more accurate understanding of alterations in Bcl-2 expression that may be present after TCDD administration.

The level of p53 expressed in hepatocytes has been associated with alterations in apoptosis in response to nongenotoxic carcinogen exposure (Christensen et al, 1998). Interestingly, in that study, TCDD did not alter apoptosis nor did it affect Bcl-2 or p53 expression. However, Rininger et al (1997), found that a single acute exposure to TCDD elevated p53 expression in the liver six days after exposure. Further, TCDD pretreatment inhibited the typical increase of p53 after exposure to DNA damaging agents in the liver (Worner and Schrenk, 1996; Paajarvi et al, 2005). Evaluation of p53 distribution showed a positive relationship between proliferative activity of the embryonic human liver and p53 expression (Lichnovsky et al, 1998). Expression of p53 clearly plays a role in hematopoiesis as well, as p53 deletions or mutations were often associated with myelodysplastic disease (Lai et al, 1995; Kurotaki et al, 2000) and inhibition of terminal differentiation of myeloid progenitors (Soddu et al, 1996). In addition, the normal expression of p53 is vital in successful hematopoietic reconstitution activity. This was shown in p53-deleted bone marrow recipients that had increased mortality after lethal irradiation even though the p53-deleted bone marrow cells engrafted recipients better than wild type donor bone marrow cells (TeKippe et al, 2003). In the current study, p53 expression was markedly inhibited, suggesting a shift away from apoptosis for the hepatocytes and a possible dysregulation of homeostasis and appropriate function of the hematopoietic compartment.

The alterations in hematopoiesis seen in this experiment are compatible with the findings of prior studies demonstrating the targeting of the hematopoietic progenitor cells, and in

particular the prolymphoid cells, by TCDD. Interestingly, the changes in the hepatocytes, not previously described, may also have a direct or indirect effect on lymphopoiesis in the developing fetus. Further investigation enumerating and identifying these hematopoietic cell populations, their potential alterations in global gene expression, as well as evaluation of the hepatocytes should be considered to more clearly understand the impact of TCDD on lymphopoiesis in the fetal mouse model.

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Table 5.1 Pooled and individual weights compared after TCDD treatment

Group	feti/litter	Pooled fetal wt	Individual fetal wt	Pooled liver wt	Individual liver wt	Ratio pooled liver wt/ pooled fetal wt
control	6.8 ± 1.5	7.9 ± 0.8	1.18 ± 0.1	0.348 ± 0.06	0.052 ± 0.011	0.044 ± 0.007
TCDD 10μg/kg	7.8 ± 1.7	9.2 ± 2.2	1.17 ± 0.1	0.505 ± 0.1*	0.066 ± 0.007*	0.056 ± 0.008*

*denotes significance at $p < 0.05$ by the Students *t* test

Table 5.2. Total cell counts compared after TCDD treatment

Group	Hepatocytes/hpf	Hematopoietic/hpf	Ratio of Hematopoietic cells/ Hepatocytes
Control	48 $\pm$ 1.83	59 $\pm$ 3.02	1.23
TCDD	47 $\pm$ 1.79	29 $\pm$ 2.61*	0.62*

*denotes significance at $p < 0.05$ by the Students *t* test

Figure 5.1A

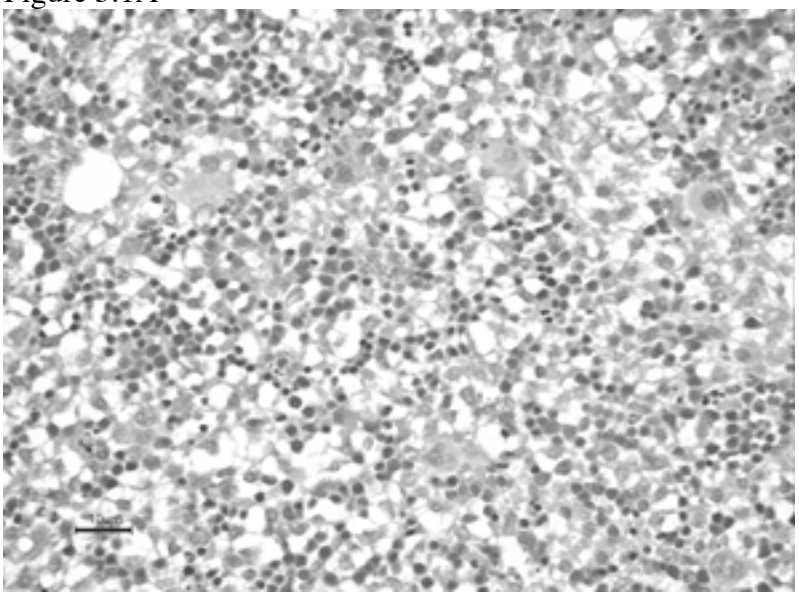

Figure 5.1B

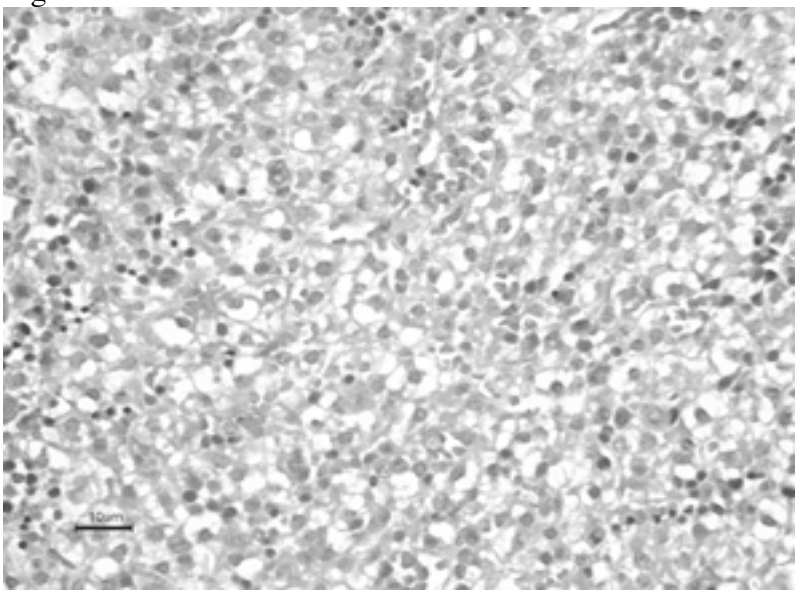

Figure 5.1

Gestation Day 18 Fetal Liver, 400x magnification. Figure 5.1A is the control liver, Figure 5.1B is the TCDD-exposed liver. Note the decrease in small, deeply staining round cells in the TCDD-exposed liver.

Figure 5.2A

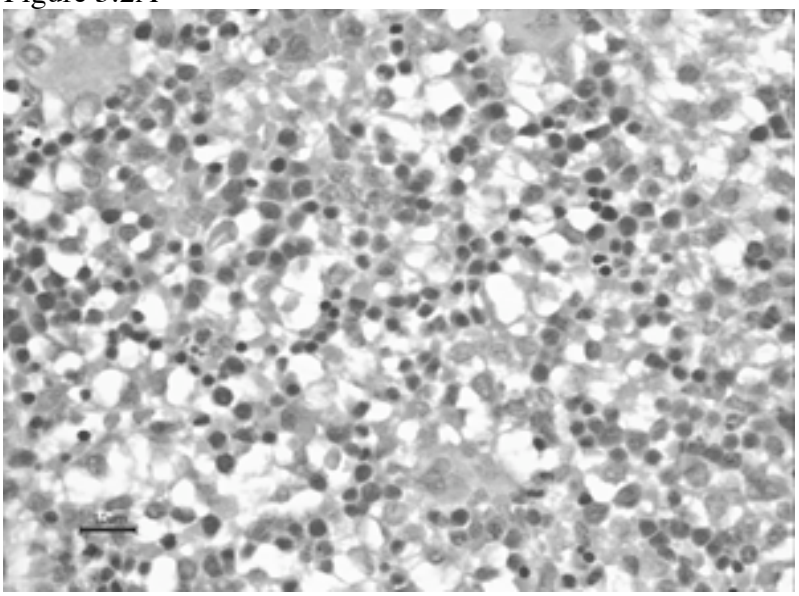

Figure 5.2B

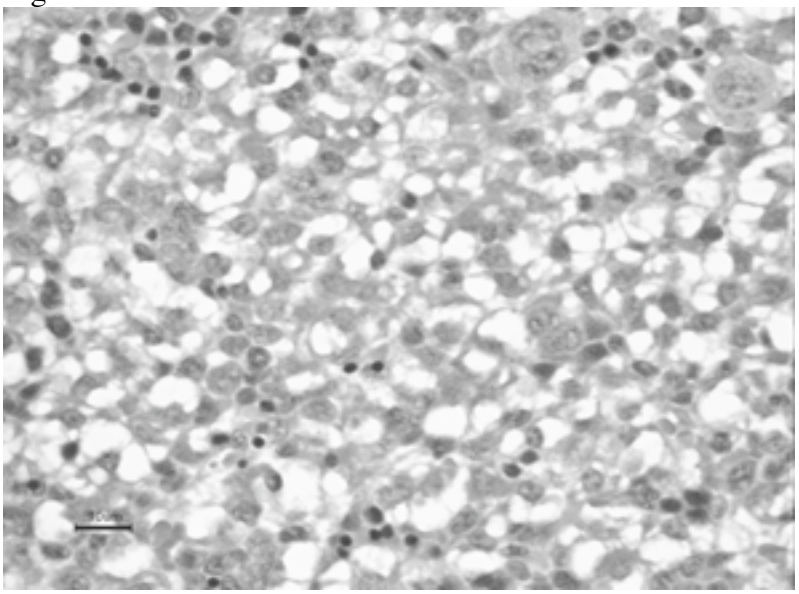

Figure 5.2

Gestation Day 18 Fetal Liver, 600x magnfication. Figure 5.2A is the control liver, Figure 5.2B is the TCDD-exposed liver. Note the cytomegaly (increased size) of the hepatocytes as well as their increased cytoplasmic staining. The decrease in small round deeply staining cells (hematopoietic stem cells/progenitor cells) is noteworthy.

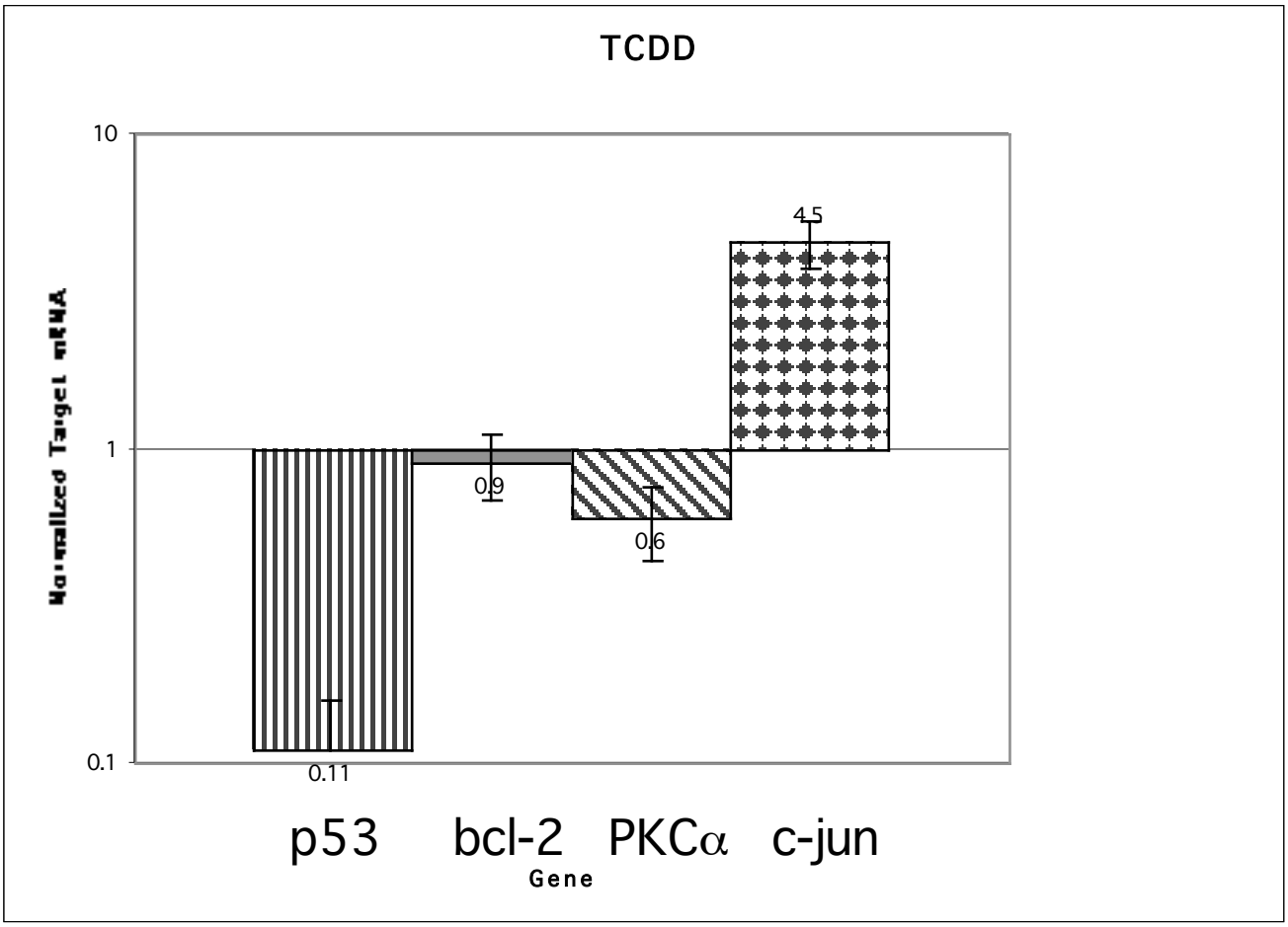

Figure 5.3

Figure 5.3. TCDD mRNA gene expression normalized to control target mRNA as described in the Materials and Methods section.