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Sustained Aryl Hydrocarbon Receptor Activity Attenuates Liver Regeneration

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ABSTRACT

In hepatocyte-derived cell lines, either loss of aryl hydrocarbon receptor (AhR) function or treatment with a persistent AhR agonist such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can disrupt G_1 phase cell cycle progression. The present study used liver regeneration to explore mechanistically how AhR activity modulates hepatocyte proliferation in vivo. Treatment of mice with 20 μg/kg TCDD 1 day before 70% partial hepatectomy (PH) resulted in a 50 to 75% suppression in liver regeneration. Impaired proliferation was not associated with changes in levels of interleukin-6 or tumor necrosis factor-α, which prime quiescent hepatocytes to enter G_1 phase. In fact, administration of TCDD 12 h after PH, a period well beyond the priming phase, still induced the G_1 arrest. Decreased proliferation in TCDD-treated mice correlated with reduced cyclin-dependent kinase-2 (CDK2) activity, a pivotal regulator of G_1/S phase transition. In contrast to observations made in cell culture, suppressed CDK2 activity was not strictly associated with increased binding of the CDK2 inhibitors $p21^{Cip1}$ or $p27^{Kip1}$. However, TCDD decreased levels of cyclin E binding to CDK2, despite normal cyclin E expression. The evidence also suggests that TCDD-induced hepatic growth arrest depends upon sustained AhR activity because transient AhR activation in response to endogenous queues failed to suppress the regenerative response. These findings establish a functional role for the AhR in regulating normal cell cycle control during liver regeneration.

The aryl hydrocarbon receptor (AhR) is a conditionally activated basic helix-loop-helix/PER/ARNT/SIM (periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded) transcription factor that mediates the toxicity of halogenated aromatic hydrocarbons and structurally related chemicals. Upon ligand binding, the receptor translocates to the nucleus and dimerizes with the AhR nuclear translocator protein, forming a complex that binds to dioxin-response elements in the promoter region of selected genes and alters transcription (Rowlands and Gustafsson, 1997). Exposure to the most potent AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) elicits an array of biological and toxic effects related to cell and/or tissue homeostasis (Mandal, 2005).

Increasing evidence indicates that the AhR influences cell cycle progression in the absence of exogenous ligand. For example, AhR-defective mouse hepatoma (Hepa 1c1c7) cells proliferate more slowly than wild-type cells. This growth inhibition occurs during G_1 phase of the cell cycle and can be reversed upon transfection with AhR cDNA (Ma and Whitlock, 1996). Moreover, mouse embryo fibroblasts from AhR nullizygous mice exhibit lower proliferation rates than mouse embryo fibroblasts from wild-type mice (Elizondo et al., 2000), providing compelling evidence that normal movement through the cell cycle requires a functional AhR.

It is noteworthy that treatment of certain cell lines with TCDD elicits changes in proliferation similar to those observed in AhR-defective or -deficient cells. In 5L rat hepatoma cells, exposure to TCDD inhibits G_1 progression (Gottlicher and Wiebel, 1991; Weiss et al., 1996). This inhibition requires the induction of $p27^{Kip1}$ to inhibit cyclin-dependent kinase 2 (CDK2) activity (Kolluri et al., 1999). In both 5L cells and mouse hepatoma cells, G_1 arrest depends on the interaction of the AhR with the retinoblastoma tumor suppressor protein (pRb) (Ge and Elferink, 1998; Puga et al., 2000; Elferink et al., 2001) and seems to involve distinct mechanisms of action (Marlowe et al., 2004; Huang and Elferink, 2005). Therefore, delays in hepatocyte cell cycle progression occur when the AhR is activated by a poorly metabolized, persistent agonist such as TCDD or when the receptor

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CDK, cyclin dependent kinase; pRb, retinoblastoma tumor suppressor protein; PH, partial hepatectomy; TNFα, tumor necrosis factor-α; IL-6, interleukin-6; UTMB, University of Texas Medical Branch; BrdU, 5-bromo-2′-deoxyuridine; RT, reverse transcription; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; C_T, threshold cycle.

is removed altogether. These seemingly contradictory findings are reconciled by the observation that impaired cell cycle progression also occurs when AhR activity is sustained in the absence of exogenous agonist by inhibiting cytochrome P4501A1 activity (Levine-Fridman et al., 2004). Thus, it seems that some level of basal or transient AhR activity, presumably through receptor interactions with endogenous ligands, promotes cell cycle progression, but prolonged AhR activity during G_1 phase progression triggers a growth arrest. This receptor-regulated activity during cell cycle progression is distinct from the observation that transgenic mice harboring a constitutively active AhR develop tumors (Moennikes et al., 2004). Tumor formation results from loss of check-point control, and the incidence of tumor development is thought to be the consequence of selecting for cells that have escaped the growth-inhibitory effect resulting from sustained AhR activity.

Relatively little is known regarding how the AhR regulates cell cycle progression in vivo. An intriguing report by Bauman et al. (1995) demonstrated that prolonged (2-week) pretreatment of rats with TCDD decreased hepatocyte proliferation after 70% partial hepatectomy (PH), implicating that AhR-mediated regulation of cell cycle progression may occur in vivo. However, the mechanism of action remains unresolved and may differ markedly from the cell culture findings, in part because liver regeneration is influenced by multiple systemically derived signals and involves nontransformed cells. To elucidate how the AhR modulates cell cycle progression in vivo, we used a mouse model of liver regeneration after PH. After resection of two thirds of the liver, remaining hepatocytes enter the cell cycle as a largely synchronized population that divides until the original organ mass is restored (Mangnall et al., 2003). Growth factors and cytokines such as TNFα and IL-6 essentially drive the otherwise quiescent hepatocytes into G_1 phase, whereas continued progression through the cell cycle is regulated primarily at the G_1/S phase transition, which is governed by the coordinated activity of CDKs (Albrecht et al., 1999). Activation of cyclin D/CDK4-6 and cyclin E/CDK2 during G_1 phase results in the phosphorylation and inactivation of pRb (Hatakeyama and Weinberg, 1995), whereas negative regulation of CDK2 activity is achieved through the binding of CDK inhibitor proteins $p27^{Kip1}$ and $p21^{Cip1}$ (Sherr and Roberts, 1995; Albrecht et al., 1998).

In the present study, TCDD was used to disrupt normal physiological AhR function and determine the contribution of sustained receptor activity to liver regeneration. Our findings demonstrate that although many of the observations made in hepatoma cell lines are recapitulated during liver regeneration, AhR activity may regulate in vivo hepatocyte proliferation by mechanisms distinct from those observed in vitro.

Materials and Methods

Animals. Female C57BL/6 mice (8–10 weeks old; The Jackson Laboratory, Bar Harbor, ME) were used for all experiments. Animals were housed in microisolator cages, maintained on a 12-h light cycle, and provided with food and water ad libitum at all times.

TCDD. TCDD (Cerilliant, Round Rock, TX) was dissolved in anisole, diluted in peanut oil, and administered via gavage at 20 μg/kg body weight. Control mice received peanut oil spiked with an equivalent amount of anisole. Mice were treated with TCDD or vehicle 24 h before surgery unless otherwise indicated. The 20 μg/kg dose of TCDD is not overtly toxic (Pohjanvirta and Tuomisto, 1994) and was selected based on a dose-response study (data not shown), in which this was the lowest dose at which consistent reproducible suppression of liver regeneration was observed.

Surgical Procedures. Mice were anesthetized with inhaled isoflurane, and PH was performed by surgically resecting 70% of the liver based on the procedure described by (Higgins and Anderson, 1931). It is noteworthy that the medial and left lateral hepatic lobes were resected individually to avoid damage to the gall bladder. The contribution of these hepatic lobes to the total liver mass was determined by wet weight to verify that removal of 70% of the liver was achieved. Control mice received a sham surgery in which the abdominal cavity was opened, and the liver was gently manipulated but not resected. All mice recovered expediently, and mortality associated with surgical procedures was less than 2%. Mice were killed postoperatively by cervical dislocation at the indicated times. All experiments were performed in compliance with the standards set by the UTMB Animal Care and Use Committee.

5-Bromo-2′-deoxyuridine Labeling. To measure proliferation in the liver, mice were injected i.p. with 50 mg/kg 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) 2 h before sacrifice. For continuous labeling studies, mice were provided drinking water containing BrdU (0.8 mg/ml) immediately after surgery, and water bottles containing BrdU were protected from light and replenished daily until mice were killed 72 h postoperatively. After sacrifice, fresh liver tissue was fixed in 10% buffered formalin for 18 h and then processed and stained for histological analysis as described below.

Histopathology/Immunohistochemistry. Slides containing liver sections were either stained with hematoxylin and eosin or processed and stained to reveal BrdU incorporation using a biotinylated anti-BrdU antibody (Invitrogen, Carlsbad, CA) followed by incubation with avidin-conjugated horseradish peroxidase and the substrate 3,3′-diaminobenzidine. Tissue processing, staining, and immunohistochemistry were performed by the Research Histopathology Core Facility at UTMB. For analysis of proliferation, the numbers of brown-stained nuclei ($BrdU^+$) were counted in three separate low-power fields per animal and expressed as a percentage of the total number of nuclei.

Measurement of TNFα and IL-6 Production. Blood was collected in heparin-coated tubes, and plasma was recovered after centrifugation. Levels of TNFα and IL-6 were measured in unpooled plasma samples using sandwich enzyme-linked immunosorbent assay kits (Pierce Biotech, Rockford, IL). The assays were performed according to the manufacturer's protocols, and all samples were run in duplicate. The lower limit of detection for the assays was 50 pg/ml.

Immunoprecipitation/Western Blotting. Frozen liver tissue was mechanically homogenized in 50 mM HEPES, pH 7.5, containing 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 7.5 mM $MgCl_2$, 7.5 mM EGTA, and the following protease/phosphatase inhibitors: 25 mM β-glycerolphosphate, 5 mM dithiothreitol, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 0.1 μg/ml leupeptin. Protein concentrations were determined using a DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). To detect cytochrome P4501A1 protein expression, liver homogenates were probed with a CYP1A1 antibody (BD Discovery Labware, Bedford, MA) by Western blotting as described below. For all other studies, liver homogenates were immunoprecipitated before Western blotting. For immunoprecipitation, 500 μl of total cellular protein (1 μg/μl) was incubated with 2 μg of rabbit or goat anti-CDK2 antibody (sc-163 or sc-163G; Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h at 4°C and then incubated for an additional hour with either protein G or protein A-Sepharose (Sigma-Aldrich). For Western blotting, Sepharose beads were washed five times in NETN buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% Nonidet P-40,

and 1 mM EDTA), fractionated by SDS-polyacrylamide gel electrophoresis on a 15% gel, and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). Membranes were blocked for 1 h at RT in 5% (w/v) dry milk in Tris-buffered saline with 0.1% (v/v) Tween 20 and then incubated for 4 h at RT or overnight at 4°C with the following primary antibodies (all purchased from Santa Cruz Biotechnology): rabbit CDK2, goat CDK2, rabbit cyclin E, rabbit cyclin A, mouse p21, and mouse p27. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT and visualized using an enhanced chemiluminescence detection method according to the manufacturer's protocol (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometry on the cytochrome P4501A1 Western blot was performed using ImageJ, a public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Quantitative Real-Time RT-PCR. Total RNA was isolated from frozen liver tissue using RNAqueous (Ambion, Inc., Austin, TX), and quantitative real-time PCR (qRT-PCR) was performed by the Sealy Center for Cancer Cell Biology and Real-Time PCR Core Facility at the University of Texas Medical Branch. Reagents included Applied Biosystems assays-by-design 20× assay mix of primers and TaqMan probes (5-carboxyfluorescein dye-labeled) for the target gene (mouse CYP1A1), and predeveloped 18S rRNA (VIC dye-labeled probe) TaqMan assay reagent for an internal control. The primers were designed to span exon-exon junctions so as not to detect genomic DNA. Validation experiments were performed to test the efficiency of the target and reference amplifications. The absolute value of the slope of log input amount versus CT was <0.1. Separate tubes (singleplex) for one-step RT-PCR were performed with 20 ng of RNA for both target gene and endogenous control using the TaqMan one-step RT-PCR master mix reagent kit (Applied Biosystems, Foster City, CA). The cycling parameters for one-step RT-PCR were reverse transcription 48°C for 30 min, AmpliTaq activation 95°C for 10 min, denaturation 95°C for 15 s, and annealing/extension 60°C for 1 min (repeat 40 times) on an ABI 7000. Triplicate CT values were analyzed in Microsoft Excel (Microsoft, Redmond, WA) using the comparative CT method as described by the manufacturer (Applied Biosystems). The amount of target (2-CT) was obtained after normalization to an endogenous reference (18S rRNA).

Kinase Assay. To measure kinase activity, 500 μg of total cellular protein was immunoprecipitated with 2 μg of CDK2 antibody as described above. Sepharose beads were washed four times in NETN buffer, once in kinase assay buffer (50 mM Tris-Cl, pH 7.5, 80 mM β-glycerolphosphate, 50 mM NaF, 20 mM EGTA, 15 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Na_3VO_4, 50 μM ATP, 5 μg/ml aprotinin, and 1 μg/ml leupeptin), and resuspended in 15 μl of kinase assay buffer containing 10 μg of histone H1 and 5 μCi [γ^{32}P]ATP (3000 Ci/mmol) (GE Healthcare). After 40 min at RT, the reaction was quenched by the addition of 15 μl of 2× SDS loading buffer. Reactions were fractionated by SDS-polyacrylamide gel electrophoresis on a 15% gel, which was subsequently dried and autoradiographed.

Statistical Analyses. Statistical analyses were performed using Prism (version 4.0; GraphPad Software, San Diego, CA). Data were evaluated either by Student's t test or by two-way analysis of variance followed by Bonferroni post hoc test. Data were considered significantly different at $p \leq 0.05$.

Results

Analysis of TCDD Toxicity in Regenerating Liver. To evaluate any overt toxic effects of TCDD treatment on the regenerating liver, a histological analysis was performed and liver wet weight was measured. PH-induced liver regeneration in vehicle-treated mice was accompanied by panlobular hydropic vacuolation of hepatic tissue (Fig. 1A). Treatment with TCDD generally increased hepatic vacuolation and also altered the integrity of hepatocyte nuclei. It is noteworthy that these effects varied considerably among animals in each treatment group. In contrast, vacuolation was not observed in liver tissue from sham-operated mice pretreated with either vehicle or TCDD, and no overt histological changes were detected in liver tissues from these mice. Consistent with the reported effects of TCDD on hepatomegaly, preoperative liver wet weight was approximately 15% greater in TCDD-treated mice compared with vehicle-treated mice, and this increase was maintained postoperatively throughout the regenerative process (Fig. 1B). Increased liver/body weight ratios in TCDD-treated mice were attributed to increased liver mass, because body weight was consistent among vehicle- and TCDD-treated mice throughout the 5-day period after surgery.

AhR Activation Suppresses Proliferation in the Regenerating Liver. The influence of AhR activation on cell cycle progression in the regenerating liver was addressed by evaluating in vivo BrdU incorporation. Thirty-six hours after PH, which is when peak DNA synthesis typically occurs in the regenerating mouse liver (Mangnall et al., 2003), BrdU incorporation confirms that approximately 60% of all hepatic

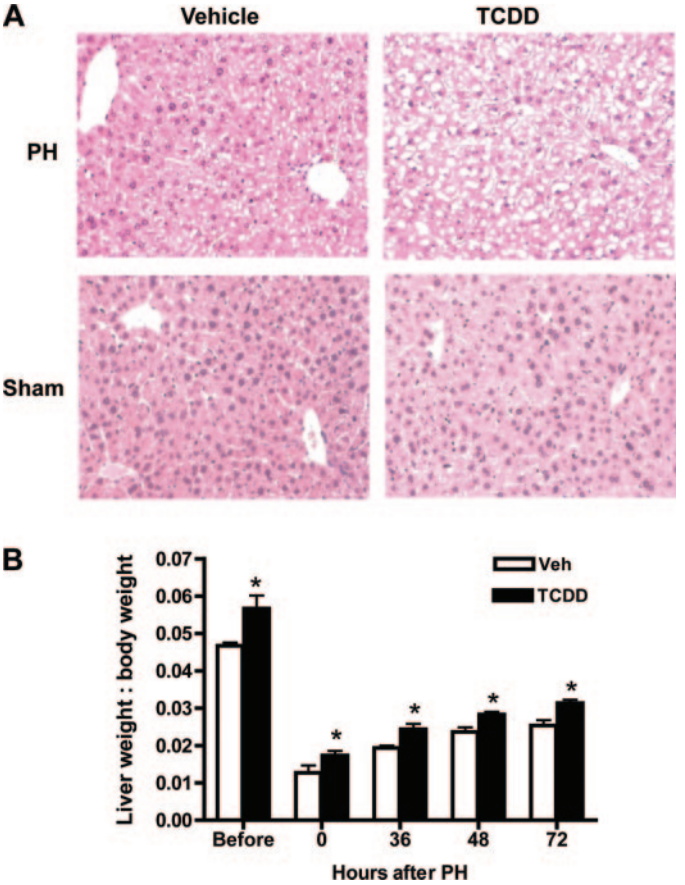

Fig. 1. Analysis of TCDD toxicity during PH-induced liver regeneration. A, representative photomicrographs (200×) of hematoxylin and eosin-stained liver tissue harvested 36 h after PH/sham surgery in mice that were pretreated for 24 h with vehicle or TCDD. B, ratio of liver weight (wet) to body weight in vehicle- and TCDD-treated mice at the indicated times after PH. For each time indicated, three to five mice were used per treatment group. Results are representative of three separate experiments. Asterisks indicate a significant difference compared with vehicle group at same time point ($p \leq 0.05$).

nuclei are in S phase in vehicle-treated mice (Fig. 2A). BrdU staining was uniform throughout the liver, consistent with a global proliferative response aimed at restoring liver cell mass. Pretreatment with TCDD resulted in approximately a 4-fold reduction in proliferation after PH (Fig. 2B). It is noteworthy that BrdU staining in these livers was also panlobular and was detected in all lobes. In contrast, BrdU incorporation was minimal (<4%) in sham-operated mice that were pretreated with either vehicle or TCDD.

To determine whether reduced proliferation in TCDD-treated mice reflected a delay in cell cycle progression instead of suppression, BrdU staining was assessed at various times throughout the regenerative process. TCDD treatment suppressed proliferation at both 36 and 48 h after PH, when peak proliferation was detected in vehicle-treated mice, but had no effect on proliferation at later times (Fig. 3A). Regeneration in TCDD-treated mice seemed to follow the same kinetics as regeneration in vehicle-treated mice. Moreover, in studies using continuous BrdU labeling, proliferation was detected in almost 100% of hepatic nuclei in vehicle-treated mice 72 h after PH (Fig. 3B). In contrast, only 50 to 60% of the nuclei in livers from TCDD-treated mice had incorporated BrdU by this time, indicating that almost half of the hepatocytes in these mice actually fail to proliferate rather than undergo a delay in cell cycle progression.

AhR Activity during the Regenerative Process. In keeping with our recent findings that sustained AhR activity is required to elicit a growth arrest (Levine-Fridman et al., 2004), we confirmed that TCDD-induced AhR activity persisted throughout the regenerative process. As shown in Fig. 4, cytochrome P4501A1 expression was induced in TCDD-pretreated mice and persisted for at least 5 days after PH, consistent with sustained AhR activity throughout the regenerative process in these mice. Also noteworthy is that removal of a substantial portion of the liver does not impair AhR activity elicited by TCDD. It is noteworthy that in vehicle-pretreated mice, cytochrome P4501A1 protein levels began to increase 36 h after PH and peaked at 72 h before tapering off. Analysis of CYP1A1 mRNA by qRT-PCR reveals that the increase in P4501A1 protein in vehicle-treated mice is preceded by a pronounced (42-fold) but transient increase in mRNA 24 h after PH (Fig. 4B). This indicates that AhR activity is transiently induced during G_1 phase of the regenerative process despite the absence of an exogenous ligand. The qRT-PCR data also confirm that the persistent expression of P4501A1 protein in livers from TCDD-treated mice is matched by sustained elevation of CYP1A1 mRNA levels.

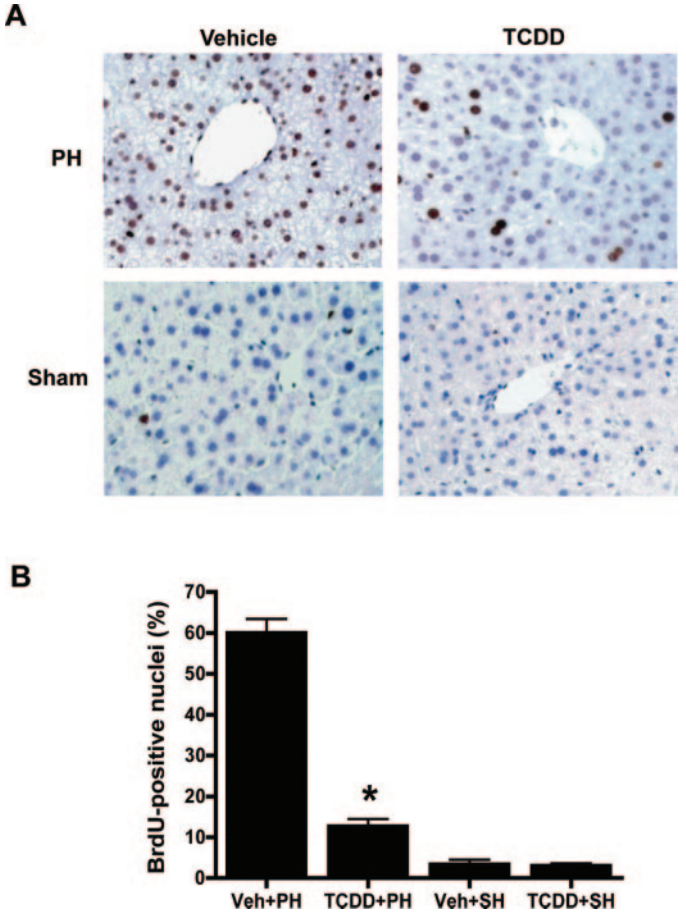

Fig. 2. Exposure to TCDD suppresses hepatocyte proliferation after PH. Mice were treated with vehicle or TCDD 24 h before PH or sham surgery. Mice were killed 36 h later, immediately after a 2-h pulse with BrdU, and liver tissue was processed and stained for immunohistochemistry. A, BrdU-positive cells are identified by the dark brown (3,3′-diaminobenzidine-stained) nuclei in the photomicrographs (200×). B, nuclei were counted in three random fields (>300 cells/field) in liver tissue from each mouse 36 h after surgery. Data represent the average percentage of $BrdU^+$ nuclei (±S.E.M.) in liver tissue from five mice per treatment group. Results are representative of six separate experiments. Asterisk indicates a significant difference compared with vehicle treatment group at same time ($p \leq 0.05$).

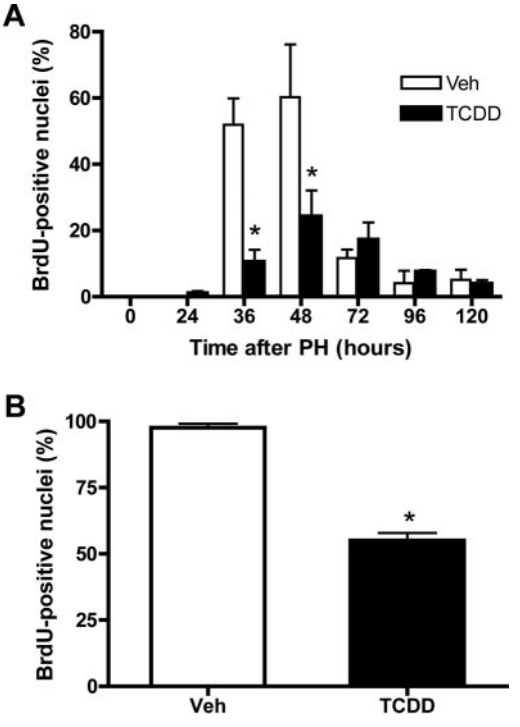

Fig. 3. Suppressed liver regeneration is not due to delayed hepatocyte proliferation in TCDD-treated mice. Mice were treated with vehicle or TCDD 24 h before PH. A, mice were pulsed for 2 h with BrdU before being sacrificed postoperatively at the indicated time. BrdU incorporation was determined as described in Fig. 2. Data represent the average percentage of $BrdU^+$ nuclei (±S.E.M.) in liver tissue from five mice per treatment group. Results are representative of three separate experiments. B, mice were continuously provided BrdU, which was added to their drinking water (0.8 mg/ml), for the 72 h immediately after PH. Data represent the average percentage of $BrdU^+$ nuclei (±S.E.M.) in liver tissue from six mice per treatment group. Asterisks indicate a significant difference compared with vehicle-treated mice that received PH ($p \leq 0.05$).

These observations complement our recent findings in cell culture (Levine-Fridman et al., 2004).

TCDD Treatment Does Not Alter the Production of IL-6 or TNFα during Liver Regeneration. Given that the initiation of liver generation depends on cytokines such as IL-6 and TNFα, which facilitate the movement of quiescent hepatocytes into G_1 phase of the cell cycle, we tested the possibility that TCDD suppressed the production of these cytokines either before or shortly after PH. In vehicle-treated mice, peak levels of IL-6 were detected 2 to 4 h after PH (Fig. 5A), whereas TNFα levels peaked 8 to 12 h after PH (Fig. 5B). Although there was a trend toward increased production of these cytokines after PH in TCDD-treated mice, this effect was not statistically significant and was not consistently observed among experiments. Before surgery, neither cytokine was detected in the plasma of mice pretreated with either TCDD or vehicle control. Likewise, cytokine production in sham-operated mice was minimally induced and remained unaltered by TCDD treatment.

Decreased Proliferation in TCDD-Treated Mice Is Not Due Solely to Alterations in Early Regenerative Processes. Based on the observation that TCDD did not impair cytokine production, we addressed the overall involvement of such early regenerative processes to the decreased proliferation observed in TCDD-treated mice. We found that when TCDD was administered 12 h after PH, well after the early "priming" phase of liver regeneration, TCDD still caused a marked decrease in proliferation, similar to that observed in mice pretreated for 24 h (Fig. 6). Likewise, a 5-fold reduction was observed in hepatic tissue from mice when TCDD was administered concomitantly with PH. The data suggest that AhR-mediated effects occur after signaling events that prompt hepatocytes out of quiescence and into the G_1 phase of the cell cycle.

CDK2 Activity Is Diminished during Liver Regeneration in TCDD-Treated Mice. Given that early events important for moving hepatocytes into G_1 phase were not altered by AhR activation, we tested the possibility that hepatocytes in TCDD-treated mice were stalling in G_1 phase. Hence, attention was directed toward the G_1 phase CDKs. An analysis of CDK4 failed to reveal TCDD-dependent alterations (data not shown), but examination of CDK2 activity exposed differences in response to TCDD treatment. In vehicle-treated mice, CDK2 phosphorylation of substrate histone H1 was readily detected 36 and 48 h after PH, whereas CDK2 activity was barely detectable 36 h after PH in TCDD-exposed mice (Fig. 7). Kinase activity in TCDD-treated mice exhibited some interindividual variability (data not shown) but generally increased at 48 h, consistent with the increase in BrdU incorporation observed between 36 and 48 h in these mice (Fig. 3A). The evidence reveals a tight correlation between diminished BrdU incorporation and reduced CDK2

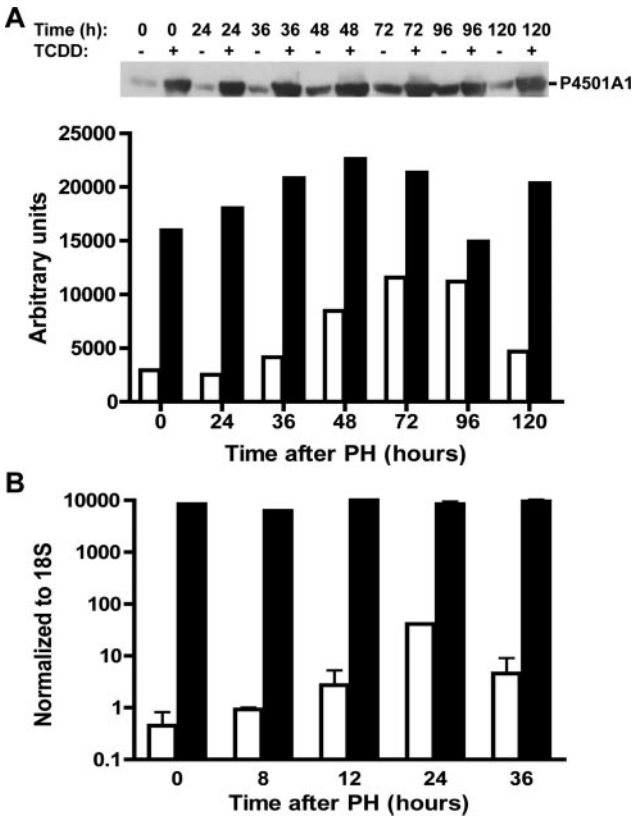

Fig. 4. AhR activity persists throughout the regenerative process in TCDD-treated mice and is transiently induced in vehicle-treated mice. Mice were treated with vehicle or TCDD 24 h before PH and sacrificed postoperatively at the indicated time. A, liver homogenates (20 μg/lane) were probed for cytochrome P4501A1 protein by Western blot, and expression was analyzed by densitometry. Data are representative of three similar experiments. B, CYP1A1 mRNA levels were measured in liver by qRT-PCR. Data are normalized to 18S and represent one mouse per treatment group. Open bars represent data from vehicle-treated mice, and solid bars indicate TCDD-treated mice.

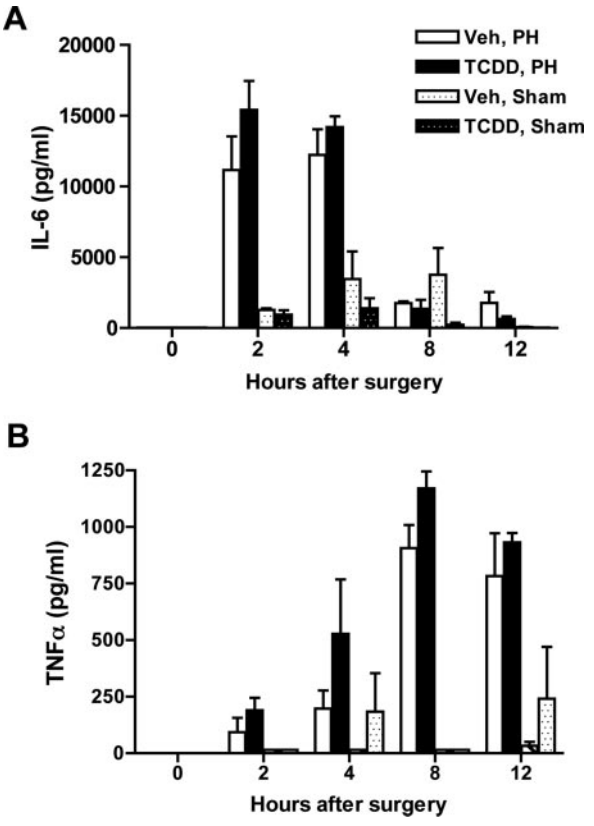

Fig. 5. TCDD treatment does not alter the production of IL-6 or TNFα during liver regeneration. Mice were treated with vehicle or TCDD 24 h before PH or sham surgery and sacrificed postoperatively at the indicated time. Data reflect the average plasma concentrations of IL-6 (A) or TNFα (B). Each treatment group consisted of five to six PH mice and three sham-operated mice. Results are representative of three to four separate experiments.

activity, demonstrating that the TCDD-induced growth arrest is linked to inhibition of G_1-phase CDK2 activity normally associated with proliferation.

AhR Activation Alters the Association of CDK2 with Cell Cycle Regulatory Proteins. To mechanistically determine why CDK2 kinase activity was impaired in TCDD-exposed mice, we conducted coimmunoprecipitation experiments to examine the association of CDK2 with cyclins E and A, regulatory proteins that bind to CDK2. CDK2/cyclin E complex formation was observed 36 and 48 h after PH (Fig. 8), consistent with cyclin E promoting CDK2 activity during cell cycle progression (Geisen and Moroy, 2002). In contrast, considerably less cyclin E was detected bound to CDK2 in the regenerating liver from TCDD-treated mice. The decrease in CDK2/cyclin E association is not reflected in total cyclin E expression, which was not noticeably affected by TCDD (data not shown). Consistent with the sequential role of cyclin E and A regulation of CDK2, cyclin A is first detected in association with the kinase at 48 h. However, reduced CDK2/cyclin A binding was observed in TCDD-treated mice, most notably at 72 h. CDK2 activity is not only a function of cyclin binding activity but is also influenced by the action of the Cip/Kip inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. Association of CDK2 with $p27^{Kip1}$ is detectable in the quiescent liver from both vehicle- and TCDD-treated mice but decreases transiently to a nearly undetectable level as cells enter G_1 phase after PH, only to recover as cells complete the first cycle of cell division. $p21^{Cip1}$ follows a reciprocal profile, because it is transiently associated with CDK2. Although $p21^{Cip1}$ and $p27^{Kip1}$ binding to CDK2 seems slightly elevated in TCDD-treated livers at 24 and 48 h, respectively, enhanced TCDD-dependent inhibitor binding to the kinase complex was neither pronounced nor consistent between animals, rendering their role in the inhibition of kinase activity uncertain. Much clearer is the marked TCDD-dependent decrease in cyclin binding as an explanation for diminished CDK2 activity.

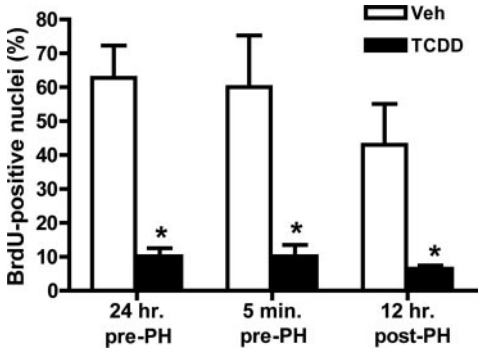

Fig. 6. Decreased proliferation TCDD-treated mice after PH is not due solely to alterations in early regenerative processes. Mice (five to eight per treatment group) were treated with vehicle or TCDD 24 h before PH, 5 min before PH, or 12 h after PH. Mice were pulsed with BrdU and killed 36 h postoperatively. Data represent the average percentage of $BrdU^+$ nuclei (±S.E.M.) in the regenerating liver. Asterisk indicates a significant difference compared with vehicle-treated mice treated at the same time point ($p \leq 0.05$).

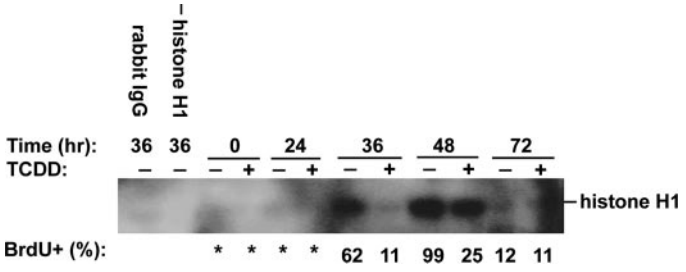

Fig. 7. CDK2 activity is diminished during liver regeneration in TCDD-treated mice. Mice were treated with vehicle or TCDD 24 h before PH and killed at the indicated time. Liver homogenates were immunoprecipitated with rabbit IgG (first lane) or CDK2 antibody (remaining lanes). Kinase activity was measured by phosphorylation of histone H1. Data represent CDK2 kinase activity in the regenerating liver from individual mice at various times after PH and is correlated with the percentage of $BrdU^+$ hepatocytes from the same mouse. An asterisk indicates that BrdU incorporation was <1%.

Discussion

Studies using hepatocyte-derived transformed cell lines demonstrate a functional role for the AhR in regulating cell cycle progression at the G_1/S phase transition. Because the establishment of immortalized cell lines relies on corrupting or bypassing cell cycle regulatory checkpoints to permit continued proliferation, it has been difficult to appreciate the relevance of such studies to receptor function in nontransformed cycling cells. Our findings using liver regeneration as a model recapitulate many of the observations made in previous cell culture studies and confirm that prolonged AhR activity can elicit a G_1 arrest in nontransformed, cycling somatic cells in vivo. However, these findings suggest that the mechanisms by which the AhR controls hepatocyte proliferation in vivo may be distinct from those identified in vitro that implicate an obligatory role for $p21^{Cip1}$ and $p27^{Kip1}$ (Kolluri et al., 1999; Barnes-Ellerbe et al., 2004).

The present findings demonstrate that TCDD treatment suppresses BrdU incorporation in the regenerating liver, suggesting that prolonged AhR activation can impair cell cycle progression into S phase and commitment to cell division. This study extends upon the previous finding by Bauman et al. (1995), in which treatment of rats with TCDD before PH reduced the percentage of cells in S phase. It is noteworthy that failure of hepatocytes to transit to S phase in our studies was not due to delayed proliferation, because cumulative BrdU incorporation was decreased 40 to 50% in TCDD-treated hepatectomized mice. We also detected only minimal apoptosis when regenerating livers from vehicle- and TCDD-treated mice were stained by terminal deoxynucleotidyl transferase dUTP nick-end labeling (data not shown), indicating that the TCDD-induced inhibition in liver regeneration is not due to increased hepatic apoptosis. The

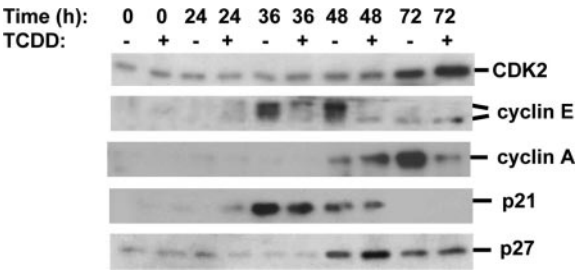

Fig. 8. AhR activation alters the association of CDK2 with cyclins E and A. Mice were treated with vehicle or TCDD 24 h before PH and sacrificed postoperatively at the indicated time. Liver homogenates were immunoprecipitated with a CDK2 antibody, and the indicated protein probed for by Western blotting. Data are representative of three separate experiments in which unpooled liver homogenates were assayed from two to three individual mice for each time point.

conclusion that S phase progression is stalled in TCDD-treated mice is further supported by the detection of reduced association of CDK2 with cyclin A, a major activating subunit of CDK2 during S phase. Taken together, our findings demonstrate that the G_1 arrest observed in transformed hepatocyte-derived cell lines treated with TCDD (Gottlicher and Wiebel, 1991; Weiss et al., 1996; Marlowe et al., 2004) is recapitulated in vivo during physiologically relevant proliferation processes.

Given the suppressive effects of TCDD on hepatocyte proliferation, it is intriguing that the mass of the remaining liver was greater in TCDD-treated mice than vehicle controls. Increased liver mass was also observed preoperatively in TCDD-treated mice, consistent with numerous reports of hepatomegaly induced by TCDD (Silkworth et al., 1989; Morris et al., 1992). Recent evidence indicates that TCDD-induced hepatomegaly occurs by an AhR-mediated mechanism that requires nuclear localization of the receptor, but the precise mechanism is unclear (Bunger et al., 2003). Although it is conceivable that the hepatomegaly observed in TCDD-treated mice results from an AhR-mediated inhibition of apoptosis in the liver, we find no evidence that apoptosis levels are altered by TCDD after PH or sham surgery. Given the increase in hydropic vacuolation that we and others have observed in livers from TCDD-treated mice (Walisser et al., 2005), it is possible that such structural changes in hepatocytes could contribute to the increase in liver mass, although additional studies would be required to address this. It is also noteworthy that vacuolation varied considerably among mice in each treatment group and did not correlate with BrdU incorporation in the liver (data not shown).

Our observation that BrdU incorporation was minimal in sham-operated mice pretreated with either vehicle or TCDD indicates that TCDD does not possess intrinsic mitogenic activity. This is consistent with other studies that demonstrate that, whereas TCDD is a potent tumor promoter, no TCDD-mediated changes in proliferation occur in the absence of a tumor-initiating agent (Pitot et al., 1980). Key mitogenic stimuli that occur early (0.5–6 h) after PH include the production of TNFα and IL-6, which essentially "drive" quiescent hepatocytes into G_1 phase. Early speculation focused on the possibility that TCDD treatment decreased the production of these cytokines immediately after PH, consistent with reports in which production of IL-6 and TNFα is variously increased or decreased, depending on the model system (Prell et al., 1995; Kerkvliet et al., 1996; Fan et al., 1997; Ito et al., 2002; Jensen et al., 2003). However, we found that neither TNFα nor IL-6 levels were suppressed in TCDD-treated mice after either PH or sham operation, indicating that these soluble mediators are not likely targets of TCDD-induced growth arrest in the regenerating liver. We also examined downstream targets of IL-6 signaling pathways and found no TCDD-mediated changes in phosphorylation of either Jak or Stat3 (data not shown). Because epidermal growth factor is also a potent liver mitogen that signals through the Stat3 protein (Cressman et al., 1995), we infer that epidermal growth factor signaling likewise is not altered by TCDD treatment. The observation that TCDD administration 12 h postoperatively still suppressed proliferation reinforces that disruption of early cytokine production or signaling is unlikely to play a significant role in the TCDD-induced growth arrest. Assuming that TCDD-mediated suppression of liver regeneration occurs independently of the priming phase, we infer that AhR activity disrupts events within G_1 phase in cycling hepatocytes. In mice, the G_1/S transition occurs 32 to 36 h after PH (Weglarz and Sandgren, 2000). Our observation that treatment with TCDD reduces CDK2 kinase activity 36 h after PH provides compelling evidence that prolonged AhR activity does indeed impair cell cycle progression through the G_1/S boundary. Although individual variation existed among mice at later time points, levels of CDK2 kinase activity closely correlated with BrdU incorporation in individual mice, strengthening the assumption that altered kinase activity in TCDD-treated mice contributes directly to suppressed hepatocyte proliferation.

Given that CDK2 activity during G_1 phase is absolutely dependent on cyclin E, it is plausible that decreased CDK2 activity in TCDD-treated mice could be explained by the decreased formation of CDK2/cyclin E complexes observed 36 and 48 h after PH. However, this contrasts with the findings made in 5L hepatoma cells, in which decreased cyclin E-dependent kinase activity did not result from decreased association of CDK2 with cyclin E (Kolluri et al., 1999). Instead, in this report, decreased kinase activity was attributed to the direct TCDD-mediated induction of $p27^{Kip1}$ and subsequent association of the inhibitor with the CDK2/cyclin E complex. Our findings did not reveal a strong correlation between TCDD treatment and $p27^{Kip1}$ expression or CDK2/$p27^{Kip1}$ (or $p21^{Cip1}$) complex formation. It is possible that CDK2 activity during liver regeneration is negatively regulated, at least in part, by the interaction between CDK2 and cyclin E. It is noteworthy that we found no evidence that TCDD altered either CDK2 or cyclin E protein levels in whole-cell lysates (date not shown), which contrasts with a recent report in which treatment of Hepa1c1c7 cells with TCDD decreased mRNA levels of both CDK2 and cyclin E (Marlowe et al., 2004). It is conceivable that the TCDD-induced decrease in cyclin E binding to CDK2 observed during liver regeneration may reside with enhanced turnover of complex-associated cyclin E. Welcker et al. (2003) recently demonstrated that CDK2- and glycogen synthase kinase-3-mediated cyclin E phosphorylation promotes SCF^{Fbw7} ubiquitin ligase-dependent degradation of the CDK-bound cyclin. Future studies examining the capacity of cyclin E phospho-site-specific mutants (refractory to ubiquitin-mediated proteolysis) designed to override the TCDD-induced growth arrest should prove insightful.

At this time, it is less clear why TCDD also reduced cyclin A binding to CDK2 at the later timepoint. The finding in C33A cells that AhR activity, in conjunction with pRb, can suppress cyclin A expression (Strobeck et al., 2000) is not supported by our observation that cyclin A levels were unaffected by TCDD. A more plausible explanation rests with the sequential nature of cyclin E and A binding to the kinase as cells progress through G_1 and S phase. The CDK2-cyclin A interaction is necessary to maintain kinase activity during S phase; hence, this complex would not be expected to form in G_1 phase-arrested cells.

The conclusion that sustained AhR activity impairs G_1/S progression is particularly intriguing in light of the data demonstrating cytochrome P4501A1 expression in the regenerating liver. In TCDD-treated mice, induction of CYP1A1 persists throughout the regenerative process. In vehicle-treated mice, transient CYP1A1 induction in the absence of

an exogenous agonist occurs 36 h after PH, which coincides with progression through G_1/S phase. Such transient activation of the AhR is insufficient for eliciting a G_1 arrest in cycling hepatocytes in vivo. Instead, it is conceivable that such transient activation is in fact required for normal cell cycle progression to occur. We have previously shown that in the absence of an exogenous agonist, AhR activity is transiently induced in serum-released 5L cells (Levine-Fridman et al., 2004). This study highlighted the negative feedback between induced P4501A1 activity and subsequent termination of AhR activity through metabolic agonist depletion. Thus, it is plausible that CYP1A1 induction during normal cell cycle progression is a mechanism directed at down-regulating receptor activity through the metabolic depletion of unidentified endogenous AhR ligands that promote cell cycle progression. Failure to engage the negative feedback results in prolonged AhR activity and induction of antiproliferative signaling.

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