Molecular Pharmacology Fast Forward. Published on November 18, 2004 as doi:10.1124/mol.104.005223

MOL5223

The Ah Receptor Predisposes Hepatocytes to Fas-Mediated Apoptosis

Kyung-Tae Park, Kristen A. Mitchell, Gengming Huang and Cornelis J. Elferink¥

Department of Pharmacology and Toxicology, University of Texas Medical Branch

¥Supported by the National Institutes of Environmental Health Sciences Grant R01ES07800 (C.J.E.), F32ES013588 (K.A.M.) and, in part, by NIEHS Grants T32ES07254 and ES06676.

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: AhR-Mediated Predisposition to Apoptosis.

All correspondence and proofs should be addressed to:
Cornelis J. Elferink, Ph.D.
Assistant Professor
Department of Pharmacology and Toxicology
University of Texas Medical Branch
301 University Boulevard
Galveston, TX 77555-1031
Ph. (409)772-9624
Fax (409)772-9642
Email coelferi@utmb.edu

Text Pages: 23
Tables: 0
Figures: 12
References: 47
Words in Abstract: 221
Words in Introduction: 749
Words in Discussion: 1,149

ABBREVIATIONS

AhR, Ah Receptor
Arnt, Ah Receptor nuclear translocator
Cyt c, Cytochrome c
efu, expression forming units
Fas, Fas receptor
FasL, Fas ligand
FBS, Fetal Bovine Serum
HRP, Horse Radish Peroxidase
3Me4NF, 3-Methoxy-4-Nitroflavone
MOI, multiplicity of infection
PARP, Poly (ADP-ribose) polymerase
PBS, Phosphate Buffered Saline
siRNA, small interfering RNA
TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TfR, Transferrin Receptor

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Liver homeostasis is achieved by the removal of diseased and damaged hepatocytes and their coordinated replacement to maintain a constant liver cell mass. Cirrhosis, viral hepatitis and toxic drug effects can all trigger apoptosis in the liver as a means to remove the unwanted cells, and the Fas "death receptor" pathway comprises a major physiological mechanism by which this occurs. The susceptibility to Fas-mediated apoptosis is, in part, a function of the hepatocyte's proteome. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor known to influence apoptosis, conceivably by regulating expression of genes involved in apoptotic signaling. In this paper we present evidence demonstrating that AhR expression and function promote apoptosis in liver cells in response to Fas stimulation. Reintroduction of the AhR into the AhR-negative BP8 hepatoma cells as well as into primary hepatocytes from AhR knockout mice, increases the magnitude of cell death in response to Fas ligand. Enhanced apoptosis correlates with increased caspase activity and mitochondrial cytochrome c release, but not with the expression of several Bcl-2 family proteins. *In vivo* studies showed that, in contrast to wild-type mice, AhR knockout mice are protected from the lethal effects of the anti-Fas Jo2 antibody. Moreover, down-regulation of the Arnt protein *in vivo* by adenovirus-mediated RNA interference to suppress AhR activity provided wild-type mice partial protection from Jo2-induced lethality.

Introduction

Apoptosis of hepatocytes is rare in healthy adult rodent livers, ranging from 1-5 apoptotic cells/ 10,000 hepatocytes (Schulte-Hermann et al., 1995). Yet certain liver disease states caused by fulminant hepatitis (representing 0.1% of all deaths in the U.S.), cirrhosis and viral hepatitis exhibit pronounced hepatocyte apoptosis induced by signaling through the Fas (CD95/APO-1) death receptor (Krammer, 1996; Feldmann, 1997; Kondo et al., 1998; Ashkenasi and Dixit, 1998; Feldmann et al., 1998; Galle and Krammer, 1998). Hepatocytes normally express high levels of Fas throughout life, which is thought to be involved in liver cell homeostasis because Fas-deficient mice develop substantial liver hyperplasia (Adachi et al., 1995).

Fas ligand (FasL) activation of the Fas receptor triggers assembly of the death-inducing signaling complex (DISC), comprising Fas, FADD/MORT1 and caspase-8. Scaffidi et al. (1998) categorized cells into two different types, distinguished by distinct Fas-mediated apoptotic signaling pathways. Type I cells are defined by a pronounced activation of caspase-8 (a member of a family of cysteine proteases activated during apoptosis) at the DISC, followed rapidly by direct caspase-3 activation. In type II cells, including hepatocytes, DISC formation and caspase-8 activation is significantly weaker than in type I cells. The caspase cascade is instead activated by the action of Bid, Bax and Bak (proapoptotic Bcl-2 family members) and Cytochrome c (Cyt c) release from the mitochondria leading to activation of the “apoptosome”, comprised of Cyt c, Apaf-1 and caspase-9 (Liu et al., 1996). Recent evidence reveals that Bid activity in hepatocytes is

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

essential for full caspase-3 activation and cleavage of inhibitor-of-apoptosis proteins (IAPs), both necessary for successful progression of the cell death program (Li et al., 2002). In contrast to the proapoptotic proteins Bid, Bak and Bax, Bcl-2 and Bcl-xL protect against apoptosis by preventing the release of Cyt c from mitochondria. Hence, Bcl-2 or Bcl-xL inhibits apoptosis only in type II cells (Strasser et al., 1995).

Ogasawara et al. (1993) demonstrated that mice injected with the anti-Fas antibody (Jo2) develop acute liver injury and die within hours of receiving the Jo2 antibody. Death results from massive hepatocyte apoptosis involving the Fas pathway. As might be expected, Fas-deficient lpr mice are refractory to the lethal dose of Jo2 antibody. Significantly, $Bid^{-/-}$ mice are also resistant to lethality induced by Jo2 indicating that Bid activity is essential for Fas-mediated apoptosis in hepatocytes (Yin et al., 1999). In contrast, $Bax^{-/-}$ mice succumb to the lethal effects of the anti-Fas antibody akin to wild-type mice (Kim et al., 2000), suggesting that Bax activity is not critical for Fas-induced hepatic apoptosis. Moreover, this latter study revealed that the actions of Bid and Bax are independent of one another, and that they may function synergistically in the mitochondrial release of Cyt c.

Numerous reports suggest that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can induce apoptosis. McConkey et al. (1988) reported that TCDD killed immature thymocytes *in vitro* by triggering apoptosis. Although reproducibility of these *in vitro* observations has proved elusive (Comment et al.,1992), Kamath et al. (1998) recently documented TCDD-induced apoptosis of thymocytes *in vivo*. Low concentrations of TCDD (100 pM-1 nM)

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

elicited apoptosis (5-10%) in mouse primary hepatocytes (Christensen et al., 1998). Similarly, TCDD induced apoptosis in the developing vasculature of fish and Xenopus hepatocytes during embryogenesis (Sakamoto et al., 1995; Cantrell et al., 1998). It is particularly noteworthy that lpr mice deficient for Fas expression are less sensitive to TCDD-mediated thymic atrophy than Fas-positive counterparts (Rhile et al., 1996; Kamath et al., 1999). Likewise, FasL defective gld mice are refractory to TCDD-induced thymic atrophy (Kamath et al., 1999). More recently Camacho et al. (2002) showed that TCDD promotes Fas-mediated apoptotic removal of activated T cells during the decline phase of an immune response. Collectively, these observations suggest that the aryl hydrocarbon receptor (AhR) plays a role in Fas mediated apoptosis.

The AhR is a cytosolic, ligand-activated transcription factor that regulates the expression of several genes in response to polycyclic and halogenated aromatic hydrocarbon ligands, such as TCDD (Okey et al., 1994; Schmidt and Bradfield, 1996). Upon ligand binding, the AhR translocates into the nucleus, dimerizes with Arnt and binds to specific DNA elements called DREs or XREs (dioxin/xenobiotic response elements) upstream of AhR-regulated genes, driving their expression. In this report we present evidence suggesting that the AhR promotes Fas-mediated apoptosis in hepatocytes in the absence of exogenous AhR agonists such as TCDD. Studies using cultured hepatoma cells, primary hepatocytes and the mouse liver *in vivo* show that AhR expression and activity, in response to endogenous signaling, predisposes liver cells to FasL-induced apoptosis.

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Materials . Fas ligand (FasL) was purchased from BD Biosciences (San Diego, CA) and the vesicular form of FasL was from United States Biological (Swampscott, MA). The caspase inhibitor benzyloxycarbonyl-valinyl-alaninyl-aspartyl-(*O*-methyl)-fluoromethylketone (z-VAD-fmk) was purchased from R&D Systems (Minneapolis, MN). 3-Methoxy-4-Nitroflavone (3Me4NF) was a gift from Dr. Gasciewicz (University of Rochester, NY). Antibodies were obtained from various commercial sources: PARP (CLONTECH), TfR (Zymed Laboratories Inc.), actin (Chemicon International), Fas, Bak, Bcl-2, Bcl-xL (Santa Cruz Biotechnology), Bax, Jo2 (Pharmingen), Bid (Santa Cruz Biotechnology), AhR (Biomol), Arnt, Cyt c (BD Biosciences).

Western Blots. Whole cell lysates were prepared by lysing cells directly in SDS-PAGE load buffer. The cytosolic fraction was prepared by washing cells once in PBS and scraping cells in 10mM Tris-HCl, 250 mM sucrose, 3 mM $MgCl_2$, pH 7.6 containing 10 µ1/ml protease inhibitor cocktail (Sigma). Cells were homogenized (Dounce) on ice and the cytosol prepared by centrifugation at 100,000xg/1h/ 4˚C. Protein was fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (BioRad) and blocked with Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk. Membranes were incubated with primary antibodies for 2-4h at room temperature followed by an incubation with HRP-conjugated secondary antibodies for 1h at room temperature, and the signal visualized using ECL (Amersham).

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

Caspase Activity Assays. Cultures of BP8 or BP8-WT cells (10^6 cells per 100 mm plates) (Elferink et al., 2001) were treated with 50 ng/mL FasL in Dulbecco's modified Eagle's medium (DMEM) for the indicated times and attached cells were harvested by scraping. Attached and detached cells were pooled and were washed once with ice-cold PBS. The cellular pellets were resuspended in 150 µl of lysis buffer [(10mM Tris-HCl, pH7.2, 142.5 mM KCl, 5 mM $MgCl_2$, 1mM EDTA, 0.25% (v/v) NP-40 with protease inhibitors [0.2mM PMSF, 0.1% aprotonin, 1 µg/ml pepstatin, 1 µg/ml leupeptin]), and stored at –80˚C until being assayed. Cell lysates were clarified by centrifugation at 12,000 x *g* for 15 min at 4˚C, and 30 µg of protein incubated for 30 min at 37˚C in a reaction volume of 90 µl containing assay buffer (100 mM HEPES, pH 7.5, 20%(v/v) glycerol, 5mM DTT, 0.5mM EDTA). Ten µl of 1mM caspase-8 (Acetyl-Leu-Glu-Thr-Asp-AFC) or 1 mM caspase-9 (Acetyl-Leu-Glu-His-Asp-AFC) substrate (Enzyme System Products) was added in a final volume of 100 µl. The caspase-catalyzed release of free AFC was quantified fluorometrically at a 400nm excitation and 505nm emission wavelength within the linear region of the assay. Caspase-8 and –9 activities are expressed as the fold change over the controls.

RT-PCR Analysis. Total RNA was isolated from 3 x10^6 BP8 or BP8-WT cells using the Chomczynski and Sacchi method (1987). First strand cDNA was generated from 1 µg of total RNA using an oligo-dT primer and Superscript II reverse transcriptase (Invitrogen). PCR (35 cycles) using Taq polymerase (QIAGEN) was performed using oligonucleotide primers for rat Fas 5'-TGAGGGTTTGGAGTTG AAGAG-3' and 5'-AGTTTT

CTTTGCACCTGCACT-3' and rat GAPDH 5'-ACCAGGGCTGCCTTCTCTTG TGACAAAGTG-3', 5'-TGAGGTCCACCACCCTGTTGCTGTAGCCAT-3' in the same reaction tube. PCR products were analyzed by fractionation on a 1% (w/v) agarose gel and visualized by ethidium bromide staining and the image captured using a gel documentation system (Alpha Innotech, San Leandro, CA).

Cell Culture and Infection BP8 cells were grown in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100ug/ml streptomycin at 37^{o}C and 5% CO_2. BP8-WT cells expressing the AhR were maintained as previously described (Elferink et a., 2001). Mouse primary hepatocytes were isolated by the collagenase perfusion from either wild-type C57Bl/6 mice or AhR knockout mice (C57Bl/6-Ahr^{tmBra}, The Jackson Lab, Bar Harbor, ME.) as described (Zaher, et al., 1998). Cells were plated at the indicated density on collagen type-I coated plates in Williams E medium containing penicillin (100 U/ml), streptomcycin (100ug/ml), insulin (5ug/ml), BSA (1mg/ml) and 10% FBS. Hepatocytes were infected with the appropriate adenovirus at a MOI of 100 at the time of plating and maintained in culture for the indicated time. Media on primary cells was changed 4 h after plating with Williams E medium containing EGF, insulin (5ug/ml), penicillin (100 U/ml), and streptomcycin (100ug/ml) and every 48 h thereafter.

Cell Sorting, Flow Cytometry and Microscopy. Cells were trypsinized, washed twice with ice-cold PBS containing 1g/L glucose and 5mM EDTA. Adenovirus-infected (GFP-positive) and uninfected (GFP-negative) cells were sorted by FACS (FACS-Vantage, BD

Biosciences) in the UTMB Flow Cytometry and Cell Sorting Core Facility. Sorted cells and trypsinized cells (for flow cytometry) were fixed in ice-cold 70% ethanol, and $3x10^6$ cells/mL stained in PBS buffer containing 50ug/mL propodium iodide and 1mg/mL RNase A for 30 min. prior to flow analysis on a FACS-Calibur flow cytometer (BD Biosciences, CA). CellQuest and ModFit LT software (Verity Software House, ME) were used to analyze subdiploid DNA content as described previously (Elferink et al., 2001). Microscopy on primary $AhR^{-/-}$ hepatocytes infected with AdGFP or AdrAhRFL was performed on live cells using a Zeiss Axiovert-200 fluorescence microscope fitted with a GFP filter. Phase contrast and fluorescence micrographs were captured using a CCD camera and Axiovision (version 3.1) software.

Construction of the Adenoviruses. Generation of AdrAhRFL was described previously (Elferink et al., 2001). AdGFP (control virus) was generated by recombination of pAdTrack-CMV with pAdEasy-1 as described by He et al. (1998). Construction of the siRNA-expressing virus (AdiArnt) is described in detail elsewhere (Huang and Elferink, 2004). All adenoviruses were maintained and purified according to the method of He et al. (1998).

Animals. All *in vivo* experiments were performed in accordance with guidelines established by the Animal Resources Program and approved by the Institutional Animal Care and Use Committee at UTMB. Female 8-week old C57Bl/6 and C57BL/6-AhR^{tmBra} AhR knockout mice (The Jackson Lab, Bar Harbor, ME) were housed in microisolator cages, maintained on a 12h light-dark cycle, and provided food and water ad libitum.

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

In Vivo Jo2 and Gene Transfer Studies. For adenovirus injections, virus was diluted in sterile PBS to 10^{10} efu/ml and administered via tail vein infusion with 100 µl (10^9 efu) per mouse. To prevent Kupffer cell-mediated elimination of virus-infected cells, mice were injected intraperitoneally with gadolinium chloride (10 mg/kg body weight; Sigma, St. Louis, MO) at 30h and 6h prior to adenovirus administration (Hardonk et al., 1992; Lieber et al., 1997). For studies using the Jo2 anti-mouse Fas, uninfected mice or mice infected for four days with the control virus (AdGFP) or siRNA virus (AdiArnt) were injected with 10 µg/ head Jo2 via tail vein infusion and monitored for survival over a 24h period.

Statistical Analysis. Data is expressed as mean ± SEM for at least three independent experiments. Two-way analysis of variance (ANOVA) was used for statistical evaluation using GraphPad Prism (4.0) software. Statistical significance (*p <0.05) is denoted by asterisks.

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

Results

The studies examining a relationship between the AhR and Fas-mediated apoptosis were initiated using the AhR-negative BP8 hepatoma cell line, and the BP8-WT line described previously (Elferink et al., 2001) that constitutively expresses the AhR. BP8 and BP8-WT cultures were treated with 50 ng/ml Fas ligand (FasL) for 6h (Fig. 1). Apoptosis was assessed by flow cytometry to measure the amount of DNA degradation depicted as subdiploid DNA (<2N). FasL-treated BP8-WT cells consistently revealed a subdiploid DNA fraction (22 ± 6%), approximately 2-fold greater than that detected in FasL-treated BP8 cells (12 ± 3%). In contrast, the subdiploid DNA content in untreated (-FasL) BP8 and BP8-WT cultures reveals few apoptotic cells (≤1.5 ± 0.8%). Moreover, FasL-induced apoptosis was completely inhibited in both cell lines by the addition of 100 μM ZVAD-fmk, a cell permeable pan-caspase inhibitor. Hence the differential increase in DNA fragmentation following FasL-stimulation provides the first indication that the AhR may contribute positively to apoptosis in liver cells. It is noteworthy that AhR activation by TCDD alone does not trigger significant apoptosis in BP8-WT cells, nor does co-administration of TCDD enhance the FasL-induced response. Likewise, treatment with the AhR antagonist 3Me4NF (Henry et al., 1999) at the time of Fas activation does not suppress the apoptotic response (Fig. 1), or affect cell viability in the absence of FasL. Collectively, these data suggest that the AhR does not directly participate in Fas-mediated cell death, but instead seems to predispose cells to die upon presentation of an apoptotic signal.

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

Qualitative measures of the difference in FasL-mediated apoptosis between the BP8 and BP8-WT cells included an analysis of poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 2). Native PARP (116 kDa) is a target for proteolytic degradation by caspase-3 yielding a characteristic 85 kDa degradation product, and serves as a biochemical marker for effector caspase activation and apoptosis. Western blotting on cell lysates from untreated and FasL treated cells reveals that PARP cleavage is significantly more pronounced in the BP8-WT cell lysates with a near complete loss of the 116kDa band (Fig. 2). Detectable PARP cleavage also occurred the BP8 cells, but at a much reduced level paralleling the flow cytometry data (Fig. 1). Some 85kDa PARP protein was also detectable in untreated BP8 cells suggestive of low-level constitutive caspase activity, however, this is not reflected by a significantly elevated level of subdiploid DNA.

Fas signaling in type II cells involves release of Cyt c from mitochondria which associates with Apaf-1 to activate caspase-9 in the apoptosome. Hence, mitochondrial Cyt c release into the cytosolic fraction provides a measure of apoptosis (Fig. 3). Western blot analysis on the cytosolic fraction from BP8 and BP8-WT cells reveals that Cyt c release is substantially greater in BP8-WT cells treated with FasL for 4h, than in the BP8 cells. Cyt c levels in the whole cell lysate confirm that the cytosolic Cyt c in FasL-treated BP8-WT cells is attributable to mitochondrial release, rather than changes in protein synthesis or turn-over. Compared with BP8-WT cells, the presence of Cyt c in the cytosol from untreated BP8 cells may be responsible for low-level caspase activity and account for the presence of some cleaved PARP protein detected in untreated cells

(Fig. 2). However, the absence of significant Cyt c release following FasL treatment is consistent with the relatively modest increase in BP8 cell apoptosis.

We also assayed for caspase-8 and 9 activity to provide a quantitative measure of FasL signaling in the BP8 and BP8-WT cells (Fig. 4). Caspase-8 is activated by the DISC upon FasL binding to Fas, and caspase-9 activity is associated with the apoptosome following mitochondrial Cyt c release. The data show that both caspases are activated in both cell lines by FasL, but that caspase-8 and 9 are significantly more active—albeit transiently—in BP8-WT cells. The difference between cell lines is most pronounced for caspase-9, consistent with the mitochondrial Cyt c release (Fig. 3). The difference in Fas signaling between the cell lines may be attributable to greater Fas receptor expression in BP8-WT cells. However, RT-PCR analysis of Fas mRNA in the cell lines reveals that steady state expression of the Fas transcript is identical between the cell lines (Fig. 5A). Likewise, Fas protein expression is equivalent in the two cell lines (Fig. 5B). Moreover, the similar increase in caspase-8 activity following 2h of FasL stimulation (Fig. 4) suggests that DISC signaling, and therefore Fas activity upon ligand binding, is not the basis for the difference seen between the BP8 and BP8-WT cells. The increase in caspase-8 activity detected at 4h in BP8-WT cells may result from a feedback mechanism in which down-stream caspases—notably caspase-6—can activate caspase-8 in the absence of further Fas signaling (Nguyen et al., 1998; Slee et al., 1999).

In light of the importance placed on the pro-apoptotic protein Bax in type II cell apoptosis, and the recent finding by Matikainen et al. (2001) that Bax expression in

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

mouse oocytes is regulated by the AhR, we examined Bax expression in the BP8 and BP8-WT cells (Fig. 6). The data reveal that the steady state Bax level is similar in both cells lines. Given this observation and the recent finding that Bax does not appear to contribute significantly to Fas induced hepatocyte apoptosis (Kim et al, 2000), it seems unlikely that the receptor's proapoptotic behavior is due to enhanced Bax activity. We also examined Bid and Bak expression given their role in Fas-mediated liver apoptosis (Yin et al., 1999; Kim et al., 2000), and the antiapoptotic proteins Bcl-2 and Bcl-xL, but found the expression of each protein to be similar between the two cell lines (Fig. 6).

In order to confirm that the increased apoptotic susceptibility of BP8-WT cells to FasL is indeed due to AhR expression rather than an unrelated consequence of generating the stable cell line, we infected BP8 cells with either an adenovirus expressing the AhR (AdrAhRFL), or a control virus (AdGFP), and examined subdiploid DNA content (Fig. 7) and Cyt c release from mitochondria in response to FasL (Fig. 8). BP8 cells were infected with the adenoviruses for 48h prior to FasL treatment for 4h (+FasL), or were left untreated (-FasL). Since all virally infected cells express GFP, it was possible to fractionate infected from uninfected cells by FACS. We analyzed apoptosis in both untreated and FasL-treated cells by measuring the subdiploid DNA content in uninfected (GFP negative) and AdGFP- or AdrAhRFL-infected (GFP positive) cells. The data show that twice as many AdrAhRFL-infected FasL-treated cells contain subdiploid DNA than the comparably treated AdGFP-infected cells (12.4 ± 4.2% vs 6.1 ± 1.3%; Fig. 7). The result also demonstrates that viral infection *per se* (i.e., in the absence of FasL) does not induce apoptosis, although a modest increase in FasL-stimulated apoptosis is detected in

AdGFP-infected cells which is independent of the AhR. However, FasL-induced apoptosis in uninfected (GFP negative) cells is identical, whether isolated from AdGFP or AdrAhRFL treated cultures. To measure Cyt c release from mitochondria, the cytosolic fraction was prepared by differential centrifugation of homogenized cells and analyzed for Cyt c content by western blotting. Consistent with the result obtained in the BP8-WT cells, FasL stimulation results in substantially more Cyt c release from the mitochondria in cells infected with AdrAhRFL than with AdGFP (Fig. 8). This strongly suggests that the apoptotic susceptibility to FasL is a function of AhR expression. FasL treatment also promotes a noticeable release of Cyt c into the cytosol in the BP8 cell infected with AdGFP. This differs from our finding in uninfected BP8 cells (see Fig. 3) and is consistent with the effect of viral infection on Fas-mediated apoptosis independent of AhR expression.

To determine whether the AhR expression status and its effect on FasL sensitivity is specific to the rat hepatoma cell line or is a more general response in liver cells, we examined whether Fas-mediated apoptosis in primary hepatocytes isolated from $AhR^{-/-}$ mice could be enhanced following ectopic AhR expression. Primary hepatocytes isolated by collagenase perfusion were infected with the AdGFP or AdrAhRFL virus and cultured for 48 h. AhR expression was monitored by western blotting (Fig. 9A). After 48 h, cultures were treated with 50 ng/ml FasL for 6h to induce Fas signaling. Cell morphology was used as an index of apoptosis scoring only infected (GFP positive) cells as either live (attached) or apoptotic (blebbed) cells in a blinded study (Fig. 9B). The combined data from two independent experiments is presented in Figure 9C, and shows

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

once again that AhR-positive cells are about twice as likely to become apoptotic than the AhR-negative counterparts following Fas activation. This result strongly supports the hypothesis that the apoptotic predisposition associated with AhR expression is a general phenomenon in liver cells. The relationship between AhR expression and hepatic apoptosis is dramatically illustrated by an *in vivo* experiment in which mice were treated with the Jo2 anti-Fas antibody, that was shown to induce massive hepatic apoptosis culminating in death (Ogasawara et al., 1993). Consistent with previous findings, we also observed that C57Bl/6 wild-type mice all died within 3-6 h of receiving 10µg/head Jo2 antibody. In contrast, three out of four $AhR^{-/-}$ mice receiving this dose of Jo2 survived, and the one mouse that died did so only after 22 h.

The *in vivo* finding was re-examined because of possible concerns that the $AhR^{-/-}$ mice are protected from the lethal dose of Jo2 antibody due to a portosystemic shunt diverting blood away from the liver (Lahvis et al., 2000). Herz and Gerard (1993) demonstrated that adenovirus administered to mice intravenously is hepatotropic and infects hepatocytes with remarkable specificity *in vivo*. Using an adenovirus (AdiArnt) expressing a small interfering RNA (siRNA) targeting Arnt protein mRNA, we sought to suppress Arnt protein expression *in vivo* and thereby interfere with AhR function in the liver. Studies using primary hepatocytes infected with AdiArnt confirm that this strategy can specifically down-regulate Arnt protein expression after 3 days, whilst cells infected with the control virus (AdGFP) retain Arnt protein expression (Fig. 11). Furthermore, using *CYP1A1* induction as a measure of AhR activity, we recently showed that the loss of Arnt protein expression completely suppresses AhR activity (Huang and Elferink,

2004). C57Bl/6 mice were treated with gadolinium chloride to suppress a Kupffer cell-mediated innate immune response to liver viral infection prior to injecting 10^9 efu of either the control AdGFP virus or AdiArnt. After four days of viral infection to provide time for Arnt protein down-regulation and changes in the hepatic proteome associated with the loss of AhR activity, mice were treated i.v. with 10μg Jo2 antibody. Wild-type mice injected with the AdiArnt virus exhibited a variable but nevertheless substantial protection from the lethal dose of Jo2 antibody (Fig. 12). The remarkable reproducibility in the time of death among mice infected with the AdGFP control virus demonstrates the consistency of this assay, and reinforces the significance of the delays in time of death due to infection with AdiArnt. Treatment with gadolinium chloride alone appeared to have only a minor effect on survival and confirms that loss of Kupffer cell function does not significantly alter the response to Jo2 antibody. Collectively, these data represent compelling evidence that AhR activity contributes positively to Fas-mediated apoptosis presumably by predisposing hepatocytes to programmed cell death, although determination of the precise mechanism responsible requires further study.

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Immune surveillance of hepatocytes is critical to liver homeostasis by ensuring that damaged and pathogen-infected cells are removed, at least in part, through Fas-mediated apoptosis. In this report we document that Fas-mediated apoptosis of liver cells is promoted by AhR expression and function. While the precise mechanism of action remains unclear, the evidence points to the AhR predisposing hepatocytes to apoptosis induced by FasL, rather than actively participating in programmed cell death following Fas stimulation. Although numerous studies describe a relationship between TCDD and apoptosis indicative of a role for the AhR in cell death, the present studies were performed in the absence of exogenous AhR agonists. Hence, the susceptibility of AhR-positive cells to Fas-signaling is attributed to receptor activity responding to endogenous signals (Levine-Fridman et al., 2004). This imputes a physiological role for the AhR, possibly by regulating the steady state expression of pro-apoptotic and/or anti-apoptotic proteins—distinct from the Bcl-2 family members examined—that render liver cells more susceptible to a death signal.

Probably the most compelling evidence for the receptor's involvement in Fas-mediated liver apoptosis comes from the survival studies with Jo2 treated mice. The wild-type mice injected with a lethal dose of the Jo2 anti-Fas antibody died within hours (Fig. 10) consistent with published observations (Ogawara et al., 1993). In contrast, three of the four AhR knockout mice survived 24h, as did the control (saline treated) mice. Moreover, the single fatality survived much longer (22h) than any of the wild-type

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

animals. While we recognize that the reported 50% portosystemic shunt in AhR$^{-/-}$ mice (Lahvis et al., 2000) may alter the pharmacodistribution of the Jo2 antibody (Fig. 10), we contend that the shunt is unlikely to explain the survival of knockout mice for the following reasons. We administered a 10 μg dose of Jo2 recognizing that as little as 1 μg Jo2 will induce lethal liver apoptosis in some mice (Redondo et al., 1996), while 3.75 μg killed 7 out of 8 animals (de la Costa et al., 1999) and 5 μg was lethal to all the mice (Takehara et al., 1999). Furthermore, the liver is the primary target for Jo2 induced apoptosis suggesting that the liver in knockout mice will eventually be exposed to the Jo2 antibody in spite of the portal shunt. This said however, we cannot absolutely exclude the possibility that AhR$^{-/-}$ mice are protected from Jo2-induced apoptotic death due to the shunt. In this context, it is worth noting that isolating primary hepatocytes from the knockout mice (Fig. 9)—which requires efficient collagenase perfusion of the liver—recovers markedly fewer isolated hepatocytes. Hence, the experiments using AdrAhRFL-infected primary hepatocytes isolated from the AhR$^{-/-}$ mice are significant because they support the *in vivo* observations without concerns about the shunt. We also demonstrated that suppressing AhR activity in a percentage of the liver cells *in vivo* by down-regulating the Arnt protein expression using RNA interference substantially protected the animals from Jo2 induced apoptosis (Fig. 12). The ability of the AdiArnt virus to only partially protect mice from Jo2 lethality is attributed to the virus infecting only a fraction of the liver—which we confirmed histologically (data not shown)—and hence, provided protection only to the subset of infected hepatocytes. We anticipate that inoculation with higher viral titers to increase hepatic infection, or reducing the lethal dose of Jo2 antibody will preferentially favor survival of AdiArnt-infected mice.

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

The experiments using cultured liver cells consistently demonstrated that AhR expression correlates with an enhanced susceptibility to Fas-mediated apoptosis. We showed this response in the stably transfected BP8-WT cells (Figs. 1-3), in the virus infected BP8 cells (Figs. 7 and 8) and virus infected primary hepatocytes from $AhR^{-/-}$ mice (Fig. 9). The enhanced release of mitochondrial Cyt c in AhR-positive cells after FasL stimulation (Figs. 3 and 8) suggests that the pro-apoptotic activity of the AhR affects the Fas signaling pathway at or before the level of Cyt c release. Comparable expression of Fas (Fig. 5) and of several Bcl-2 family members (Fig. 6) in BP8 and BP8-WT cells, suggest that these are not AhR target genes in liver cells. These measures of protein expression do not address their subcellular distribution or function however, and it remains a formal possibility that the difference in apoptotic susceptibility is tied to the activity of one or other of these proteins. However, the data show that the apoptotic susceptibility of BP8-WT cells is not simply due to altered expression of Fas or one (or more) of the Bcl-2 family proteins known to participate in Fas-mediated hepatocyte apoptosis. The finding that Bax gene expression is unaffected by AhR expression in the hepatocytes is particularly significant given the recent observation that the Bax gene is a receptor target gene in mouse oocytes in response to PAHs (Matikainen et al., 2001). Given that Bax does not appear to contribute significantly to Fas induced hepatocyte apoptosis (Kim et al, 2000), despite its role in oocyte apoptosis (Matikainen et al., 2001), it seems unlikely that AhR's proapoptotic behavior seen in liver cells is attributable to enhanced Bax activity. Future studies will examine whether the activity of Bid, Bak, or the anti-apoptotic proteins Bcl-2 and Bcl-xL can account for the pro-apoptotic response.

Assessment of caspase-8 and 9 activity in BP8 and BP8-WT cells following FasL stimulation (Fig. 4) offers some insights: the induction of caspase-8 activity within 2 h of FasL treatment is equivalent in both cell lines suggesting that recruitment of the DISC components and subsequent caspase-8 activation is not influenced by AhR activity. This differs from the AhR-dependent increase in both Fas and caspase-8 expression detected in Jurkat T cells (Ito et al., 2004), and may represent a distinction between Type I and Type II cells. We do however, detect a significant enhancement of caspase-8 activity in the BP8-WT by 4 h and speculate that this reflects a feedback response involving down-stream caspases (e.g. caspase-6) that trigger further caspase-8 activation to amplify the signaling cascade (Nguyen et al., 1998; Slee et al., 1999). Activation of caspase-9, which is inherently a latent response, is also significantly greater in BP8-WT cells following FasL stimulation for 4h, which heralds the increased incidence of PARP cleavage (Fig. 2) and DNA degradation (Fig. 1) in these cells. Collectively, these data suggest that AhR function in liver cells affects the Fas pathway down-stream of the DISC, but up-stream of caspase-9 activation, by a mechanism promoting Cyt c release from the mitochondria. Reiners and Clift (1999) showed that AhR function in the mouse Hepa 1 cells also enhanced C_2-ceramide induced apoptosis by a mechanism not dependent on direct AhR participation. C_2-ceramide action is distinct from Fas signaling however, apparently inducing mitochondrial Cyt c release through a mechanism involving disruption of the mitochondrial transmembrane potential and subsequent mitochondrial lysis (Richter and Ghafourifar, 1999). Hence, the implication is that AhR pro-apoptotic activity seems to

affect mitochondrial Cyt c release, possibly by regulating the expression or function of either pro- or anti-apoptotic proteins that control Cyt c release.

References

Adachi M, Suematsu S, Kondo, T, and Nagata S (1995). Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. *Nature Genet.* **11:**294-300.

Ashkenasi A, and Dixit VM (1998). Death receptors: signaling and modulation. *Science*, **281:**1305-1308.

Camacho IA, Nagarkatti M, and Nagarkatti PS (2002) 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces Fas-dependent activation-induced cell death in superantigen-primed T cells. *Arch.Toxicol.* **76:**570-580.

Cantrell SM, Joy-Schlezinger J, Stegeman JJ, Tillitt DE, and Hannink M (1998). Correlation of 2,3,7,8-tetracholodibenzo-p-dioxin-induced apoptotic cell death in the embryonic vasculature with embryogenesis. *Toxicol. Appl. Pharmacol.* **148:**24-34.

Chomezynski P,and Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:**156-159.

Christensen JG, Gonzalez AJ, Cattley RC,and Goldsworthy TL(1998). Regulation of apoptosis in mouse hepatocytes and alteration of apoptosis by nongenotoxic carcinogens. *Cell Growth & Diff.* **9:**815-825.

Comment CE, Blaylock BL, Germolec DR, Pollack PL, Kouchi Y, Rosenthal GJ, and Luster IM (1992). Thymocyte injury after *in vitro* chemical exposure: Potential mechanisms for thymic atrophy. *J. Pharmacol. Exp. Ther*. **262:**1267-1273.

de la Coste CA, Fabre M, McDonell N, Porteu A, Gilgenkrantz H, Perret C, Kahn A, and Mignon A (1999) Differential protective effects of Bcl-xL and Bcl-2 on apoptotic liver injury in transgenic mice. *Am.J.Physiol* **277:**G702-G708.

Elferink CJ, Ge N-L,and Levine A (2001). Maximal Ah Receptor Activity Depends on an Interaction with the Retinoblastoma Protein. *Mol. Pharmacol.* **59:**664-673.

Feldmann G. (1997). Liver apoptosis. *J. Hepatol*. **26:**1-11.

Feldmann G, Lamboley C, Moreau A, and Bringuier A(1998). Fas-mediated apoptosis of hepatic cells. *Biomed. & Pharmacother.* **52:**378-385.

Galle PR and Krammer PH (1998). CD95-Induced apoptosis in human liver. *Seminars in Liver Disease,* **18:**141-151.

Hardonk MJ, Dijkhuis FW, Hulstaert CE,and Koudstaal J (1992). Heterogeneity of rat liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. *J Leukoc Biol* **52:**296.

He T-C, Zhou S, Da Costa LT, Yu J, Kinzler, KW, and Vogelstein B (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA*, **95:**2509-2514.

Henry EC, Kende AS, Rucci G,Totleben MJ, Willey JJ, Dertinger SD, Pollenz RS, Jones JP, and Gasiewicz TA (1999). Flavone antagonists bind competitively with 2,3,7, 8-tetrachlorodibenzo-p-dioxin (TCDD) to the aryl hydrocarbon receptor but inhibit nuclear uptake and transformation. *Mol Pharmacol*, **55:**716-725.

Herz J and Gerard, RD (1993). Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc. Natl. Acad. Sci. USA.* **90:**2812-2816.

Huang G and Elferink CJ (2004). Multiple Mechanisms are Involved in Ah Receptor-Mediated Cell Cycle Arrest. (submitted to *Mol. Pharmacol*.).

Ito T, Tsukumo S, Suzuki N, Motohashi H, Yamamoto M, Fujii-Kuriyama Y, Mimura J, Lin TM, Peterson RE, Tohyama C, Nohara K. (2004). Constitutively active arylhydrocarbon receptor induces growth inhibition of Jurkat T cells through changes in the expression of genes related to apoptosis and cell cycle arrest. *J Biol Chem*.**279:**25204-25210

Kamath, AB, Xu H, Nagarkatti PS, and Nagarkatti M (1998). Evidence for the induction of apoptosis in thymocytes by 2,3,7,8-tetrachlorodibenzo-p-dioxin *in vivo*. *Toxicol. Appl. Pharmacol.* **142:**367-377.

Kamath AB, Camacho I, Nagarkatti PS, Nagarkatti M (1999). Role of Fas-Fas ligand interactions in 2,3,7,8-tetrachlorodibenzo- p-dioxin (TCDD)-induced immunotoxicity: increased resistance of thymocytes from Fas-deficient (lpr) and Fas ligand-defective (gld) mice to TCDD-induced toxicity. *Toxicol. Appl. Pharmacol.* **160:**141-155

Kim TH, Zhao Y, Barber MJ, Kuharsky DK, Yin XM. (2000) Bid-induced cytochrome c release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. *J Biol Chem.* **275:**39474-39481

Kondo T, Suda T, Fukuyama H, Adachi M, and Nagata S (1998). Essential roles of the Fas ligand in the development of hepatitis. *Nature Med*. **3:**409-413.

Krammer PH (1996). The CD95 (APO-1/Fas) receptor/ligand system: death signals and diseases. *Cell Death Differ*. **3:**159-160.

Lahvis GP, Lindell SL, Thomas RS, McCuskey RS, Murphy C, Glover E, Bentz M, Southard J, and Bradfield CA. (2000) Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice. *Proc Natl Acad Sci U S A.* **97:**10442-10447.

Levine-Fridman A, Chen L and Elferink CJ (2004). Cytochrome P450IA1 Promotes G1 Phase Cell Cycle Progression by Controling Ah Receptor Activity. *Mol. Pharmacol.* **65:**461–469.

Li S, Zhao Y, He X, Kim TH, Kuharsky DK, Rabinowich H, Chen J, Du C, and Yin XM (2002) Relief of extrinsic pathway inhibition by the Bid-dependent mitochondrial release of Smac in Fas-mediated hepatocyte apoptosis. *J.Biol.Chem.* **277:**26912-26920.

Lieber A, He CY, Meuse L, Schowalter D, Kirillova I, Winther B, and Kay. MA (1997). The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J. Virol*. **71:**8798-8807.

Liu X, Kim CN, Yang J, Jemmerson R, and Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86:**147-157.

Luo X, Budihardjo I, Zou H, Slaughter C, and Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* **94:**481-490.

Matikainen T, Perez GI, Jurisicova A, Pru JK, Schlezinger JJ, Ryu HY, Laine J, Sakai T, Korsmeyer SJ, Casper RF, Sherr DH, Tilly JL. (2001) Aromatic hydrocarbon

receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nat Genet*. **28:**355-60

McConkey, DJ, Hartzell P, Duddy SK, Hakansson H, and Orrenius S (1988). 2,3,7,8-Tetrachlorodibenzo-p-dioxin kills immature thymocytes by calcium mediated endonuclease activation. *Science* **242:**256-259.

Okey AB, Riddick DS, and Harper PA (1994). Molecular biology of the aromatic hydrocarbon (dioxin) receptor. *Trends in Pharmacol. Sci.* **15:**226-232.

Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S (1993). Lethal effect of the anti-Fas antibody in mice. *Nature*, **364:**806-809

Nguyen, M, Branton PE Roy S, Nicholson DW, Alnemri E.S. Yeh W-C, Mak, TW, Shore GC (1998). E1A-induced Processing of Procasphase-8 Can Occur Independently of FADD and Is Inhibited by Bcl-2. *J. Biol Chem.* **273**:33099-33102.

Redondo C, Flores I, Gonzalez A, Nagata S, Carrera AC, Merida I, and Martinez A (1996) Linomide prevents the lethal effect of anti-Fas antibody and reduces Fas-mediated ceramide production in mouse hepatocytes. *J.Clin.Invest* **98**:1245-1252.

Reiners JJ and Clift RE (1999). Aryl hydrocarbon receptor regulation of ceramide-induced apoptosis in murine hepatoma 1c1c7 cells. *J. Biol. Chem.* **274:**2502-2510.

Richter C, and Ghafourifar P (1999) Ceramide induces cytochrome c release from isolated mitochondria. *Biochem Soc Symp*. **66:**27-31

Rhile MJ, Nagarkatti M, and Nagarkatti PS (1996). Role of Fas apoptosis and MHC genes in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity of T cells. *Toxicol.* **110:**153-167.

Sakamoto MK, Mima S, and Tanimura T (1995). A morphological study of liver lesions in Xenopus larvae exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with special reference to apoptosis of hepatocytes. *J. Environ. Pathol. Toxicol. Oncol.* **14:**69-82.

Scaffidi C, Fulda, S., Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH and Peter ME (1998). Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* **17:**1675-1687.

Schmidt JV, Su G H-T, Reddy JK, Simon MC, and Bradfield CA (1996). Characterization of a murine Ahr null allele: animal model for the toxicity of halogenated dioxins and biphenyls. *Proc. Natl. Acad. Sci. USA,* **93:**6731-6736.

Schulte-Hermann R, Bursch W, and Grasl-Kraupp B (1995). Active cell death (apoptosis) in liver biology and disease. Boyer, J.L., and Ockner, R.K. eds.) *Progress in liver disease.* **13:**1-35.

Slee EA, Harte MT, KlucK RM, Wolf BB, Casiao CA, Newmeyer DD, Wang H-G, Reed JC, Nicholson DW, Alnemri ES, Green DR, and Martin SJ, (1999). Ordering the Cytochrome c-initiated Caspase Cascade: Hierarchical Activation of Caspases-2, -3, -6, -7, -8, and -10 in a Caspase-9-dependent Manner. *The Journal of Cell Biology*. **144**:281-292.

Strasser A, Harris AW, Huang DC, Krammer PH, and Cory S (1995) Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J.* **14:**6136-6147.

Takehara T, Hayashi N, Tatsumi T, Kanto T, Mita E, Sasaki Y, Kasahara A, and Hori M (1999) Interleukin 1ß protects mice from Fas-mediated hepatocyte apoptosis and death. *Gastroenterology* **117:**661-668.

Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA, Korsmeyer SJ. (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* **400:**886-891

Zaher H, Fernandez-Salguero PM, Letterio J, Sheikh MS, Fornace AJ Jr, Roberts AB, Gonzalez FJ. (1998) The involvement of aryl hydrocarbon receptor in the activation of transforming growth factor-beta and apoptosis. *Mol Pharmacol*. **54:**313-321.

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1: Flow cytometry on FasL treated BP8 and BP8-WT cells. Subconfluent cultures of BP8 and BP8-WT cells were untreated (-FasL) or treated with 50 ng/ml FasL (+FasL) for 6h in the absence (-) or presence (+) of 10 nM TCDD, 1 μM 3Me4NF or 100 μM ZVAD-fmk. Adherent cells were trypsinized and pooled with the detached cells, followed by washing in PBS and 70% ethanol fixation. Cells ($3x10^6$/ml) were stained with propidium iodide and subjected to flow cytometry to measure DNA content. DNA fragmentation associated with apoptosis is detected as subdiploid (<2N) DNA. Apoptosis (given as percentages) was measured by quantitating the DNA in the subdiploid region depicted by the bracket using MODfit software. The data represents the mean ± SEM from independent experiments (the number of independent experiments for each treatment is given in parentheses).

Figure 2: Analysis of PARP cleavage in BP8 and BP8-WT cells. Total cell lysates (20 μg) were prepared from untreated (-) and 50 ng/ml FasL/6h treated (+) cultures and analyzed by Western blotting for the PARP protein. PARP is detected as the 116kDa mature form and 85kDa caspase cleavage product. Transferrin receptor (TfR) is analyzed as a loading control.

Figure 3: Cyt c release from mitochondria in FasL-treated BP8 and BP8-WT cells. Whole cell lysate (WCL) and cytosol fractions (20 µg) from untreated (-) and 50 ng/ml FasL/4h treated (+) cultures were analyzed by Western blotting for the Cyt c protein. Actin is used as a loading control.

Figure 4: Caspase-8 and –9 activity in BP8 and BP8-WT cells. Subconfluent cultures of BP8 (open squares) and BP8-WT (solid squares) cells were treated with 50 ng/ml FasL for 2-6 h, after which cell lysates were prepared for caspase-8 (top) and caspase-9 (bottom) enzyme activity using isozyme-specific fluorogenic substrates as described in experimental procedures. Caspase activity is presented as change (fold increase) in activity in FasL-treated cells relative to untreated cells (control). The data represent the mean ± SEM from at least three independent experiments performed in triplicate. *$p<0.05$, using a two-way ANOVA.

Figure 5: Fas expression in BP8 and BP8-WT cells. A) Total RNA was isolated from BP8 and BP8-WT cells and used for first strand synthesis with an oligo-dT primer and RTase. First-strand cDNA was used in PCR with primers directed to rat Fas and GAPDH (as a control). PCR products were fractionated on an agarose gel and the DNA visualized by ethidium bromide staining. B) Whole cell lysates were fractionated by SDS-PAGE, transferred to Hybond and subjected to western blotting for Fas protein and TfR (loading control).

Molecular Pharmacology Fast Forward. Published on November 18, 2004 as DOI: 10.1124/mol.104.005223
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6: Expression of proapoptotic and antiapoptotic Bcl-2 family proteins in BP8 and BP8-WT cells. Total cell lysates (20 µg) from untreated BP8 and BP8-WT cultures were fractionated by SDS-PAGE, transferred to Hybond and probed for the indicated proteins. The Bid protein detected is the uncleaved 22kDa precursor. TfR is included as a loading control.

Figure 7: AhR expression in BP8 cells by adenoviral infection promotes FasL induced apoptosis. Subconfluent BP8 cell cultures were infected for 48 h with AdGFP or AdrAhRFL at an MOI of 50 to obtain a population of both infected and uninfected cells in the same culture. Cultures were untreated (-FasL) or treated with 50 ng/ml FasL/6h (+FasL). Cells were trypsinized, pooled with detached cells and sorted by FACS into uninfected (-GFP) and virus infected (+GFP) populations for both AdGFP and AdrAhRFL treated cultures. Cells were fixed in 70% ethanol, stained with propidium iodide and subjected to flow cytometry. The subdiploid (<2N) DNA content was determined for each sorted population using ModFit software. The data represent the mean ± SEM of at least four independent experiments.

Figure 8: Cyt c release from mitochondria in adenovirus infected BP8 cells. Subconfluent cultures of asynchronous BP8 cells were infected with the AdGFP (control) or AdrAhRFL (wild-type AhR) adenovirus for 48 h at an MOI of 100. Cultures were treated with PBS (-) or 50 ng/ml FasL/4h (+) and the cytosol (20 µg) analyzed by western blotting for Cyt c and actin (used as a loading control).

Figure 9: AhR expression in primary hepatocytes from AhR$^{-/-}$ mice increases FasL-induced apoptosis. Primary hepatocytes were isolated by collagenase perfusion from AhR$^{-/-}$ mice. Cells were infected with AdGFP or AdrAhRFL at a MOI of 100 and maintained in culture for 48h before treatment with PBS (-) or 50 ng/ml FasL (+) for 6h. A) AhR expression in AdrAhRFL infected cells was confirmed by western blotting on total cell lysates (10 µg) from PBS-treated cells. TfR is a loading control. B) Infected cells (GFP positive) were scored in a blinded study as live (i.e., attached) or apoptotic (i.e., detached and blebbed). Phase contrast and fluorescence micrographs depict the cell morphology used to score cells. C) The bar graph data represents the mean ± SEM from two independent experiments in which at least 200 cells were scored for each condition.

Figure 10: Survival of wild-type and AhR$^{-/-}$ mice following treatment with the Jo2 antibody. C57BL/6 wild-type mice (n=4; solid squares) and C57BL/6-Ahr^{tmBra} AhR knockout mice (n=4; solid circles) were injected intravenously with 10µg/head Jo2 antibody in 50 µl PBS and followed for up to 24h. Wild-type control mice (n=2; open squares) were injected with 50 µl PBS. Percent survival was plotted against time of death for each animal until 24 h.

Figure 11: Adnovirus-mediated siRNA suppresses Arnt protein expression in murine primary hepatocytes. Primary hepatocytes isolated from a C57Bl/6 mouse by collagenase perfusion were infected for 0-5 days with AdiArnt expressing a siRNA specific for the murine Arnt mRNA. At the indicated times, cells were collected and total cell lysates fractionated by SDS-PAGE and analyzed by western blotting for the Arnt protein and actin (loading control).

Figure 12: Suppressing Arnt protein expression protects mice from Jo2-induced lethality. C57Bl/6 mice were injected intraperitoneally with $GdCl_3$ (10 mg/kg body weight) at 30h and 6h prior to adenovirus administration. Adenovirus was administered (10^9 efu in100 µl PBS) via tail vein infusion and four days later mice were injected with 10 µg/head Jo2 via tail vein infusion and monitored for survival. Treatments included $GdCl_3$ alone (squares, n=3), $GdCl_3$ and AdGFP (diamonds, n=5), $GdCl_3$ and AdiArnt (triangles, n=4). The result represents the collective survival data for all mice from two independent experiments.

Figure 1

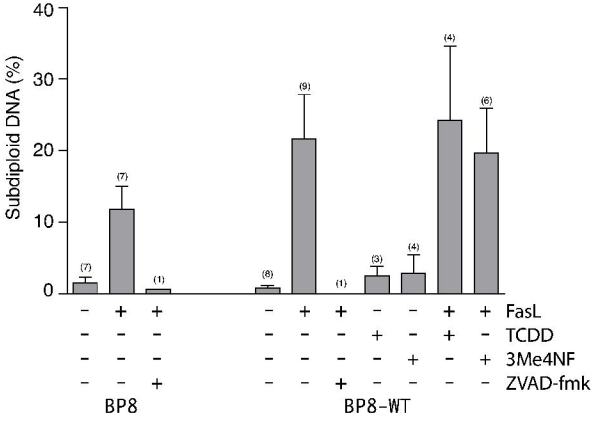

Subdiploid DNA (%)
40
30
20
10
0
(7)
(7)
(1)
(8)
(9)
(1)
(3)
(4)
(4)
(6)
FasL
TCDD
3Me4NF
ZVAD-fmk
BP8
BP8-WT

Figure 2

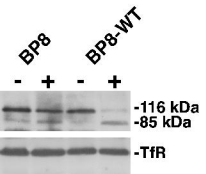


Figure 3

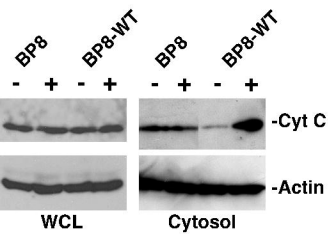


Figure 4

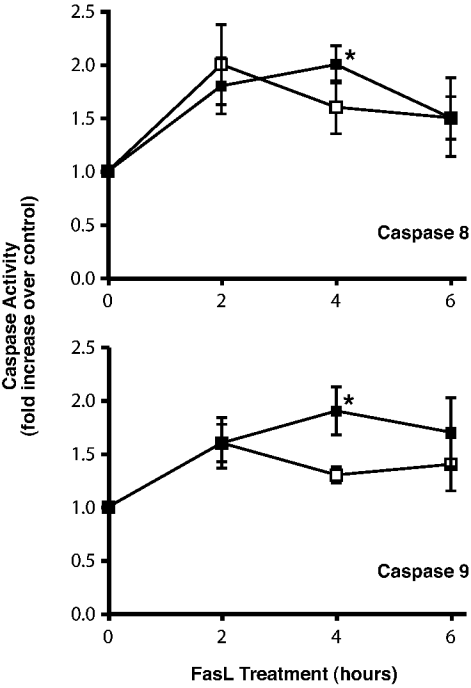

Caspase Activity
(fold increase over control)
2.5
2.0
1.5
1.0
0.5
0.0
*
Caspase 8
0
2
4
6
2.5
2.0
1.5
1.0
0.5
0.0
*
Caspase 9
0
2
4
6
FasL Treatment (hours)

Figure 5

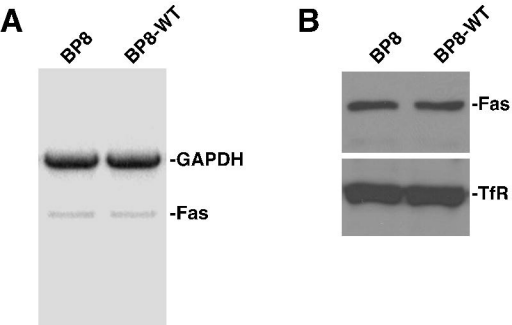


Figure 6

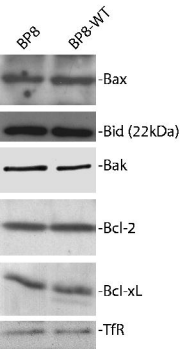


Figure 7

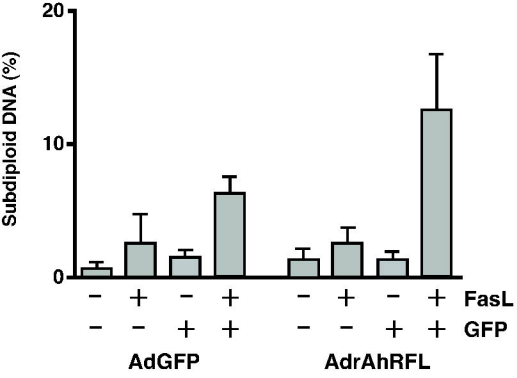


Figure 8

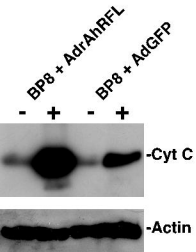
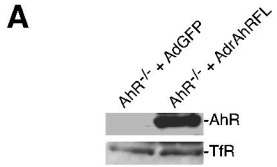
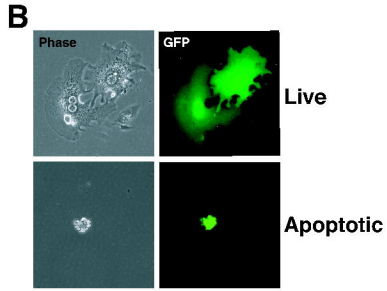


Figure 9


A
AhR-/- + AdGFP
AhR-/- + AdrAhRFL
-AhR
-TfR


B
Phase
GFP
Live
Apoptotic

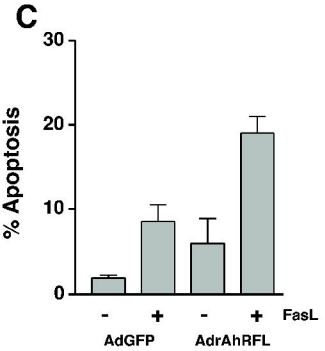

C
% Apoptosis
30
20
10
0
- + - + FasL
AdGFP
AdrAhRFL

Figure 10

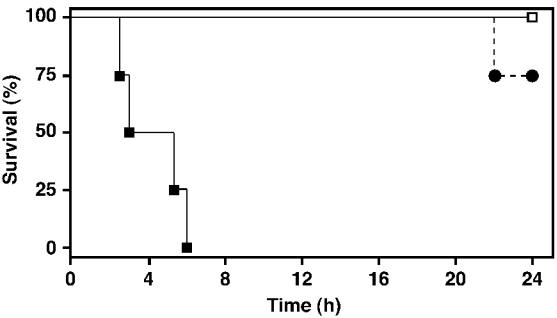


Figure 11

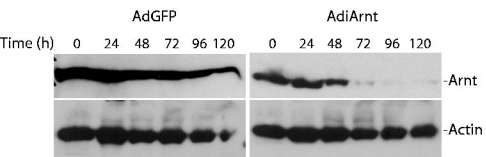

AdGFP
AdiArnt
Time (h)
0
24
48
72
96
120
0
24
48
72
96
120
-Arnt
-Actin

Figure 12