

# The Aryl Hydrocarbon 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, Galveston, Texas*

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## 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 of removing 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 the expression of genes involved in apoptotic signaling. In this article, 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 aryl hydrocarbon receptor nuclear translocator protein in vivo by adenovirus-mediated RNA interference to suppress AhR activity provided wild-type mice partial protection from Jo2-induced lethality.

Apoptosis of hepatocytes is rare in healthy adult rodent livers, ranging from 1 to 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 United States), cirrhosis, and viral hepatitis exhibit pronounced hepatocyte apoptosis induced by signaling through the Fas (CD95/APO-1) death receptor (Krammer, 1996; Feldmann, 1997; Ashkenasi and Dixit, 1998; Feldmann et al., 1998; Galle and Krammer, 1998; Kondo et al., 1998). Hepatocytes normally express high levels of Fas throughout life, which is believed 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), com-

prising 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”, composed of Cyt *c*, Apaf-1, and caspase-9 (Liu et al., 1996; Luo et al., 1998). Recent evidence reveals that Bid activity in hepatocytes is essential for full caspase-3 activation and cleavage of inhibitor-of-apoptosis proteins, 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).

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**ABBREVIATIONS:** FasL, Fas ligand; AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; Cyt *c*, cytochrome *c*; efu, expression-forming units; 3Me4NF, 3-methoxy-4-nitroflavone; m.o.i., 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; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DISC, death-inducing signaling complex; ZVAD-fmk, benzyloxycarbonyl-valinyl-alaninyl-aspartyl-(O-methyl)-fluoromethylketone.

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. It is significant that *Bid*<sup>-/-</sup> 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*<sup>-/-</sup> 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 to 1 nM) elicited apoptosis (5–10%) in mouse primary hepatocytes (Christensen et al., 1998). Likewise, TCDD induced apoptosis in the developing vasculature of fish and *Xenopus laevis* 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. Together, 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 et al., 1996). Upon ligand binding, the AhR translocates into the nucleus, dimerizes with Arnt, and binds to specific DNA elements called dioxin- or 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, predispose liver cells to FasL-induced apoptosis.

## Materials and Methods

**Materials.** FasL was purchased from BD Biosciences (San Jose, CA), and the vesicular form of FasL was from United States Biological (Swampscott, MA). The caspase inhibitor benzoyloxycarbonyl-valinyl-alaninyl-aspartyl-(*O*-methyl)-fluoromethylketone (ZVAD-fmk) was purchased from R&D Systems (Minneapolis, MN). 3-Methoxy-4-nitroflavone (3Me4NF) was a gift from Dr. Gasciewicz

(University of Rochester, Rochester, NY). Antibodies were obtained from various commercial sources: poly(ADP-ribose) polymerase (PARP) (BD Biosciences Clontech, Palo Alto, CA); transferrin receptor (TfR) (Zymed Laboratories, South San Francisco, CA); actin (Chemicon International, Temecula, CA); Fas, Bak, Bcl-2, and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA); Bax and Jo2 (BD Pharmingen, San Diego, CA); *Bid* (Santa Cruz Biotechnology); AhR (BIOMOL Research Laboratories, Plymouth Meeting, PA); and Arnt and *Cyt c* (BD Biosciences Clontech).

**Western Blots.** Whole-cell lysates were prepared by lysing cells directly in SDS-PAGE loading buffer. The cytosolic fraction was prepared by washing cells once in PBS and scraping cells in 10 mM Tris-HCl, 250 mM sucrose, and 3 mM MgCl<sub>2</sub>, pH 7.6, containing 10 μl/ml protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cells were homogenized (Dounce) on ice, and the cytosol was prepared by centrifugation at 100,000g for 1 h at 4°C. Protein was fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and blocked with Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dry milk. Membranes were incubated with primary antibodies for 2 to 4 h at room temperature followed by an incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, and the signal was visualized using enhanced chemiluminescence (Amersham Biosciences Inc., Piscataway, NJ).

**Caspase Activity Assays.** Cultures of BP8 or BP8-WT cells (10<sup>6</sup> cells/100-mm plates) (Elferink et al., 2001) were treated with 50 ng/ml FasL in Dulbecco's modified Eagle's medium 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 [10 mM Tris-HCl, pH 7.2, 142.5 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 0.25% (v/v) Nonidet P-40 with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin)] and stored at -80°C until being assayed. Cell lysates were clarified by centrifugation at 12,000g for 15 min at 4°C, and 30 μg of protein was 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, 5 mM dithiothreitol, and 0.5 mM EDTA]. Ten microliters of 1 mM caspase-8 (acetyl-Leu-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin) or 1 mM caspase-9 (acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin) substrate (Enzyme System Products, Livermore, CA) was added in a final volume of 100 μl. The caspase-catalyzed release of free 7-amino-4-trifluoromethyl coumarin was quantified fluorometrically at a 400 nm excitation and 505 nm emission wavelength within the linear region of the assay. Caspase-8 and caspase-9 activities are expressed as the fold change over the controls.

**Reverse Transcriptase-PCR Analysis.** Total RNA was isolated from 3 × 10<sup>6</sup> 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, Carlsbad, CA). PCR (35 cycles) using Taq polymerase (QIAGEN, Valencia, CA) was performed using oligonucleotide primers for rat Fas (5'-TGAGGGTTTGGAGTTGAAGAG-3' and 5'-AGTTTCTTTGCACCTGCACT-3') and rat GAPDH (5'-AC-CAGGGCTGCCTTCTCTGTGACAAAGTG-3' and 5'-TGAGGTC-CACCACCCTGTTGCTGTAGCCAT-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 was captured using a gel documentation system (Alpha Innotech, San Leandro, CA).

**Cell Culture and Infection.** BP8 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO<sub>2</sub>. BP8-WT cells expressing the AhR were maintained as described previously (Elferink et al., 2001). Mouse primary hepatocytes were isolated by the collagenase perfusion from either wild-type C57BL/6 mice or AhR knockout mice (C57BL/6-Ahr<sup>tmBra</sup>, The Jackson Labo-

ratory, Bar Harbor, ME) as described previously (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), streptomycin (100  $\mu$ g/ml), insulin (5  $\mu$ g/ml), bovine serum albumin (1 mg/ml), and 10% fetal bovine serum. Hepatocytes were infected with the appropriate adenovirus at an m.o.i. of 100 at the time of plating and maintained in culture for the indicated time. Media on primary cells were changed 4 h after plating with Williams' E medium containing epidermal growth factor, insulin (5  $\mu$ g/ml), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) and every 48 h thereafter.

**Cell Sorting, Flow Cytometry, and Microscopy.** Cells were trypsinized and washed twice with ice-cold PBS containing 1g/l glucose and 5 mM EDTA. Adenovirus-infected (GFP-positive) and uninfected (GFP-negative) cells were sorted by FACS (FACS-Vantage; BD Biosciences) in the University of Texas Medical Branch Flow Cytometry and Cell Sorting Core Facility. Sorted cells and trypsinized cells (for flow cytometry) were fixed in ice-cold 70% ethanol, and  $3 \times 10^6$  cells/ml were stained in PBS buffer containing 50  $\mu$ g/ml propidium iodide and 1 mg/ml RNase A for 30 min before flow analysis on a FACS-Calibur flow cytometer (BD Biosciences). CellQuest and ModFit LT software (Verity Software House, Topsham, ME) were used to analyze subdiploid DNA content as described previously (Elferink et al., 2001). Microscopy on primary AhR<sup>-/-</sup> hepatocytes infected with AdGFP or AdAhRFL was performed on live cells using a Zeiss Axiovert-200 fluorescence microscope fitted with a GFP filter (Carl Zeiss Inc., Thornwood, NY). Phase contrast and fluorescence micrographs were captured using a charge-coupled device camera and Axiovision (version 3.1) software (Carl Zeiss Inc.).

**Construction of the Adenoviruses.** Generation of AdAhRFL 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 small interfering RNA (siRNA)-expressing virus (AdiArnt) is described in detail elsewhere (Huang and Elferink, 2005). All adenoviruses were maintained and purified according to the method described by 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

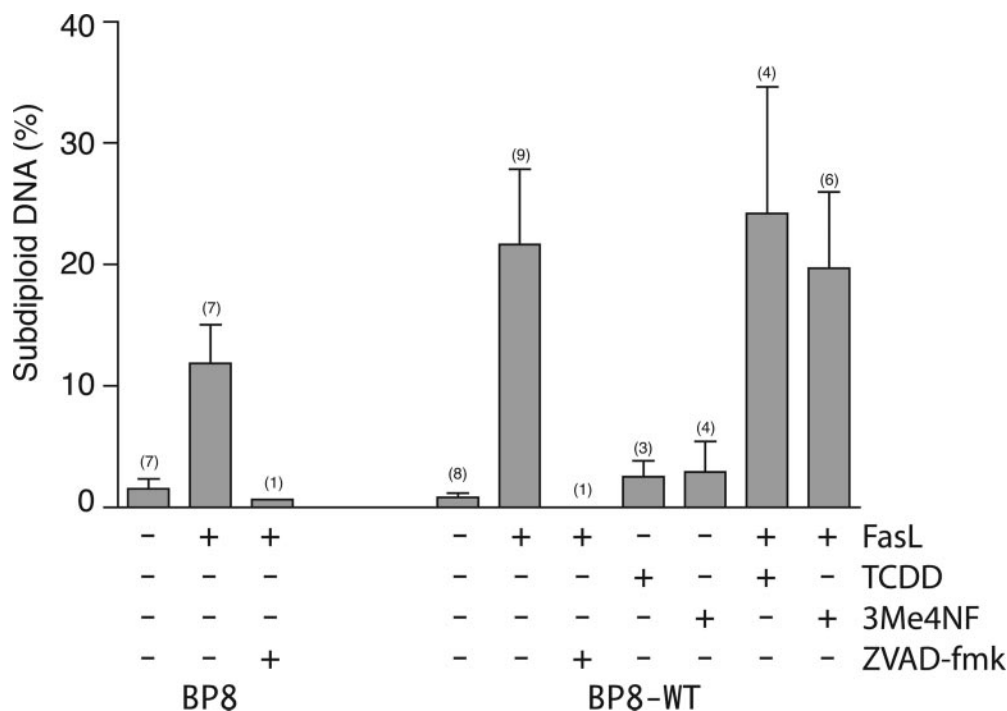
the University of Texas Medical Branch. Female 8-week-old C57BL/6 and C57BL/6-AhR<sup>tmBra</sup> AhR knockout mice (The Jackson Laboratory) were housed in microisolator cages, maintained on a 12-h light/dark cycle, and provided food and water ad libitum.

**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  $\mu$ l ( $10^9$  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-Aldrich) at 30 and 6 h before adenovirus administration (Hardonk et al., 1992; Lieber et al., 1997). For studies using the Jo2 anti-mouse Fas, uninfected mice or mice infected for 4 days with the control virus (AdGFP) or siRNA virus (AdiArnt) were injected with 10  $\mu$ g/head Jo2 via tail vein infusion and monitored for survival over a 24-h period.

**Statistical Analysis.** Data are expressed as mean  $\pm$  S.E.M. for at least three independent experiments. Two-way analysis of variance was used for statistical evaluation using GraphPad Prism (version 4.0) software (GraphPad Software Inc., San Diego, CA). Statistical significance ( $p < 0.05$ ) is denoted by asterisks.

## 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 FasL for 6 h (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 ( $22 \pm 6\%$ ), approximately 2-fold greater than that detected in FasL-treated BP8 cells ( $12 \pm 3\%$ ). In contrast, the subdiploid DNA content in untreated ( $-$ FasL) BP8 and BP8-WT cultures reveals few apoptotic cells ( $\leq 1.5 \pm 0.8\%$ ). Moreover, FasL-induced apoptosis was completely inhibited in both cell lines by the addition of 100  $\mu$ M ZVAD-fmk, a cell-permeable



**Fig. 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 6 h in the absence ( $-$ ) or presence ( $+$ ) of 10 nM TCDD, 1  $\mu$ M 3Me4NF, or 100  $\mu$ M ZVAD-fmk. Adherent cells were trypsinized and pooled with the detached cells, followed by washing in PBS and 70% ethanol fixation. Cells ( $3 \times 10^6$ /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 quantifying the DNA in the subdiploid region depicted by the bracket using ModFit software. The data represent the mean  $\pm$  S.E.M. from independent experiments (the number of independent experiments for each treatment is given in parentheses).



pan-caspase inhibitor. Hence, the differential increase in DNA fragmentation after 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 coadministration 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. Together, 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.

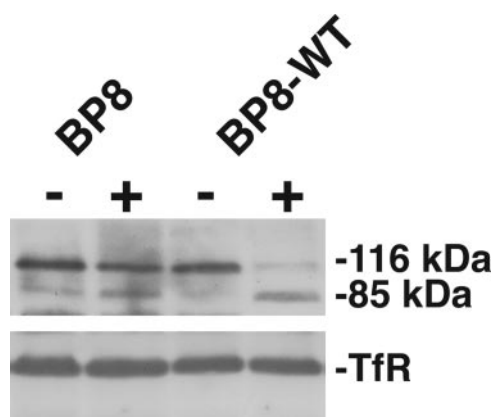
Qualitative measures of the difference in FasL-mediated apoptosis between the BP8 and BP8-WT cells included an analysis of PARP cleavage (Fig. 2). Native PARP (116 kDa) is a target for proteolytic degradation by caspase-3, yielding a characteristic 85-kDa degradation product, and serves as a biochemical 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 116-kDa band (Fig. 2). Detectable PARP cleavage also occurred in the BP8 cells but at a much reduced level paralleling the flow cytometry data (Fig. 1). Some 85-kDa 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 the 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 4 h 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 to changes in protein synthesis or turnover. Compared with BP8-WT cells, the presence of Cyt *c* in the cytosol from untreated BP8 cells may be responsible for low-level caspase

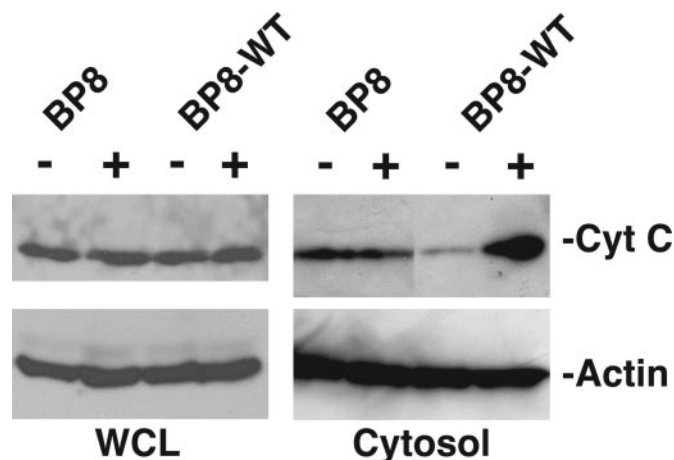
activity and account for the presence of some cleaved PARP protein detected in untreated cells (Fig. 2). However, the absence of significant Cyt *c* release after FasL treatment is consistent with the relatively modest increase in BP8 cell apoptosis.

We also assayed for caspase-8 and caspase-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 after mitochondrial Cyt *c* release. The data show that both caspases are activated in both cell lines by FasL, but that caspases-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, reverse transcriptase-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 after 2 h 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 4 h in BP8-WT cells may result from a feedback mechanism in which downstream caspases—namely 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 proapoptotic protein Bax in type II cell apoptosis and the recent finding by Matikainen et al. (2001) that Bax expression in 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 cell lines. Given this observation and the recent finding that Bax does not seem to contribute significantly to Fas-induced hepatocyte apoptosis (Kim et al., 2000), it seems unlikely that the receptor's proapoptotic behavior is caused by enhanced Bax activity. We also examined Bid and Bak expression, given their roles in Fas-mediated liver apoptosis (Yin et al., 1999; Kim et



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

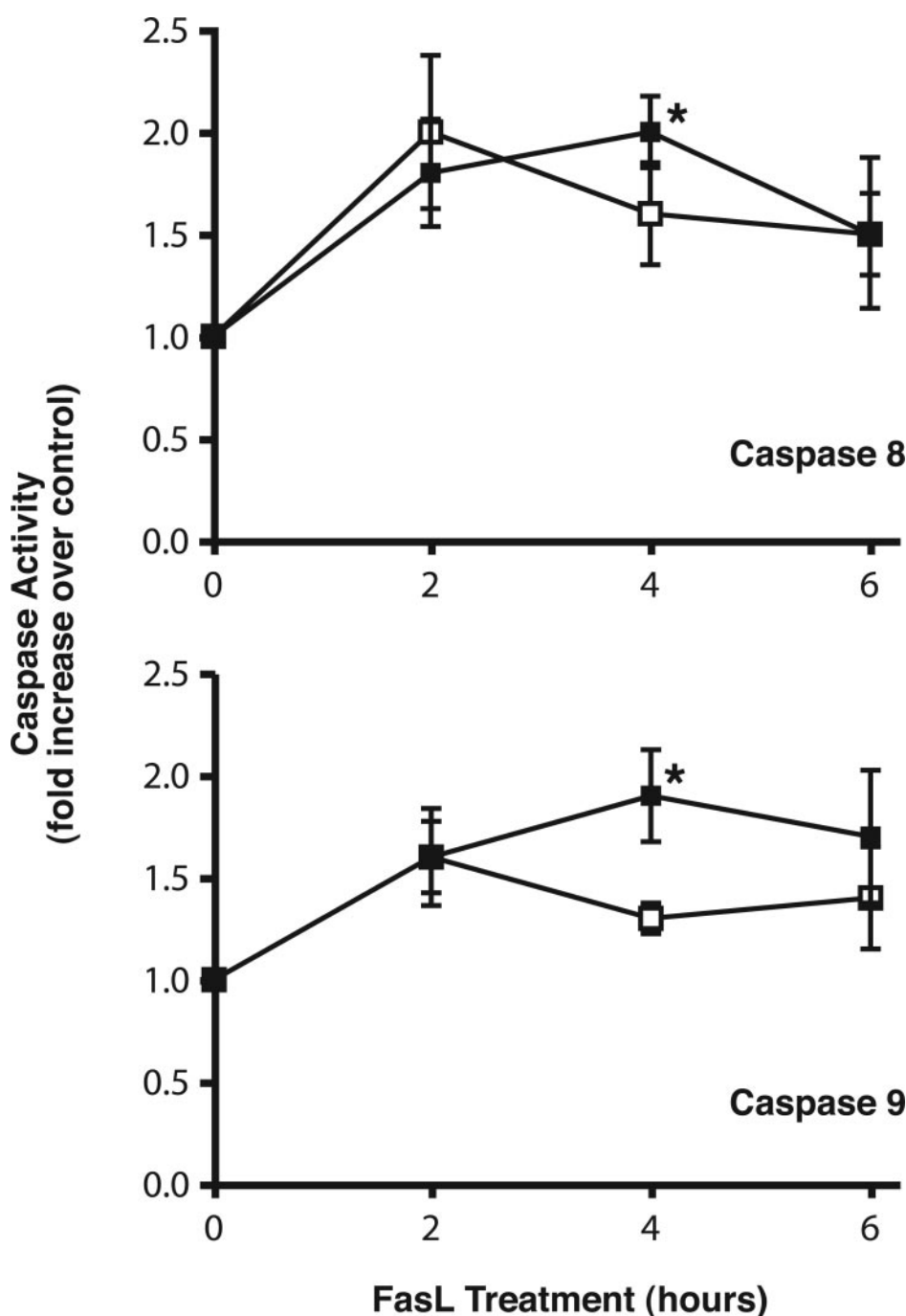


**Fig. 3.** Cyt *c* release from mitochondria in FasL-treated BP8 and BP8-WT cells. Whole-cell lysate (WCL) and cytosol fractions (20  $\mu$ g) from untreated (–) and 50 ng/ml FasL/4 h-treated (+) cultures were analyzed by Western blotting for the Cyt *c* protein. Actin is used as a loading control.

al., 2000), and the antiapoptotic proteins Bcl-2 and Bcl-xL, but we found the expression of each protein to be similar between the two cell lines (Fig. 6).

To confirm that the increased apoptotic susceptibility of BP8-WT cells to FasL is indeed caused by AhR expression rather than an unrelated consequence of generating the stable cell line, we infected BP8 cells with either an adenovirus expressing the AhR (AdAhRFL) 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 48 h before FasL treatment for 6 h (+FasL) or were left untreated (–FasL). Because 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 AdAhRFL-infected (GFP-positive) cells. The data show that twice as many AdAhRFL-infected FasL-treated cells contain subdiploid DNA than the comparably treated AdGFP-infected cells ( $12.4 \pm 4.2$  versus  $6.1 \pm 1.3\%$ ) (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 AdAhRFL-treated cultures. To measure Cyt *c* release from mitochondria, the cytosolic fraction was



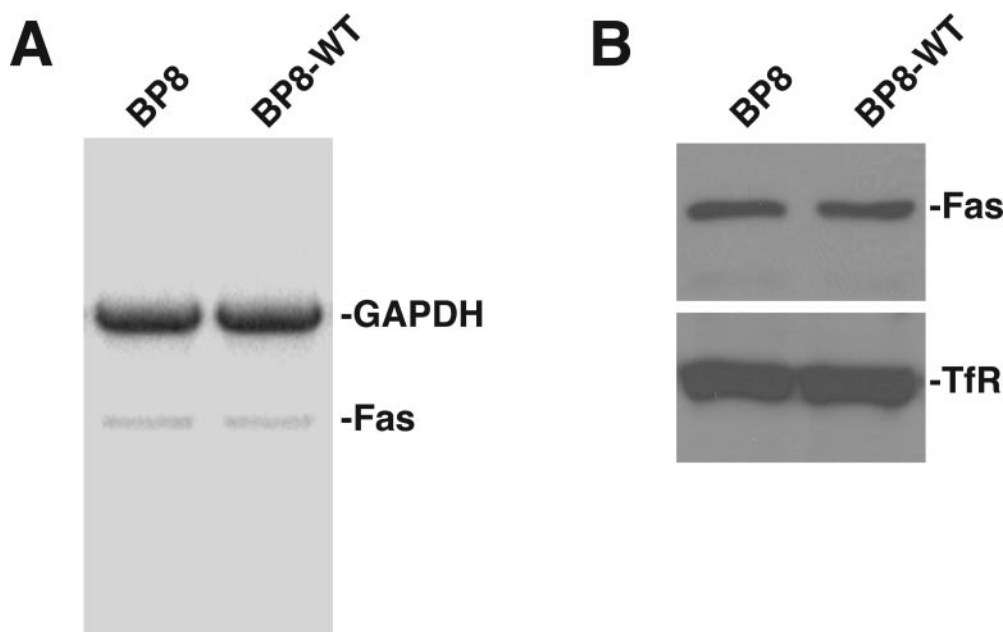
**Fig. 4.** Caspase-8 and -9 activity in BP8 and BP8-WT cells. Subconfluent cultures of BP8 (□) and BP8-WT (■) cells were treated with 50 ng/ml FasL for 2 to 6 h, after which cell lysates were prepared for caspase-8 (top) and caspase-9 (bottom) enzyme activity using isozyme-specific fluorogenic substrates as described under *Materials and Methods*. Caspase activity is presented as change (fold increase) in activity in FasL-treated cells relative to untreated cells (control). The data represent the mean  $\pm$  S.E.M. from at least three independent experiments performed in triplicate. \*,  $p < 0.05$  using a two-way analysis of variance.

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 (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<sup>-/-</sup> mice could be enhanced after 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 6 h 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 are presented in Fig. 9C and show once again that AhR-positive cells are approximately twice as likely to become apoptotic as the AhR-negative counterparts after 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, which was shown to induce massive hepatic apoptosis (Fig. 10), culminating in death (Ogasawara et al., 1993). Consistent with previous findings, we also observed that C57BL/6 wild-type mice all died within 3 to 6 h of receiving 10 µg/head Jo2 antibody. In contrast, three of four AhR<sup>-/-</sup>

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<sup>-/-</sup> mice are protected from the lethal dose of Jo2 antibody because of 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 an 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, whereas 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, 2005). C57BL/6 mice were treated with gadolinium chloride to suppress a Kupffer cell-mediated innate immune response to liver viral infection before injecting 10<sup>9</sup> efu of either the control AdGFP virus or AdiArnt. After 4 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 intravenously with 10 µg of 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 caused by infection with AdiArnt. Treatment with gadolinium chloride alone seemed 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. Together, these data represent compelling evidence that AhR activity contributes positively to Fas-me-



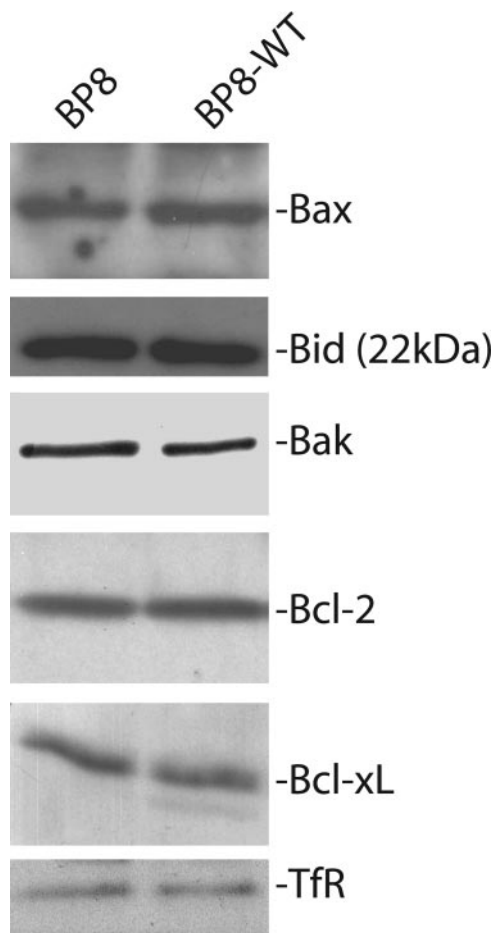
**Fig. 5.** Fas expression in BP8 and BP8-WT cells. A, total RNA was isolated from BP8 and BP8-WT cells and used for first-strand synthesis with an oligo(dT) primer and RTase. First-strand cDNA was used in PCR with primers directed to rat Fas and GAPDH (as a control). PCR products were fractionated on an agarose gel, and the DNA was 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).

diated apoptosis presumably by predisposing hepatocytes to programmed cell death, although determination of the precise mechanism responsible requires further study.

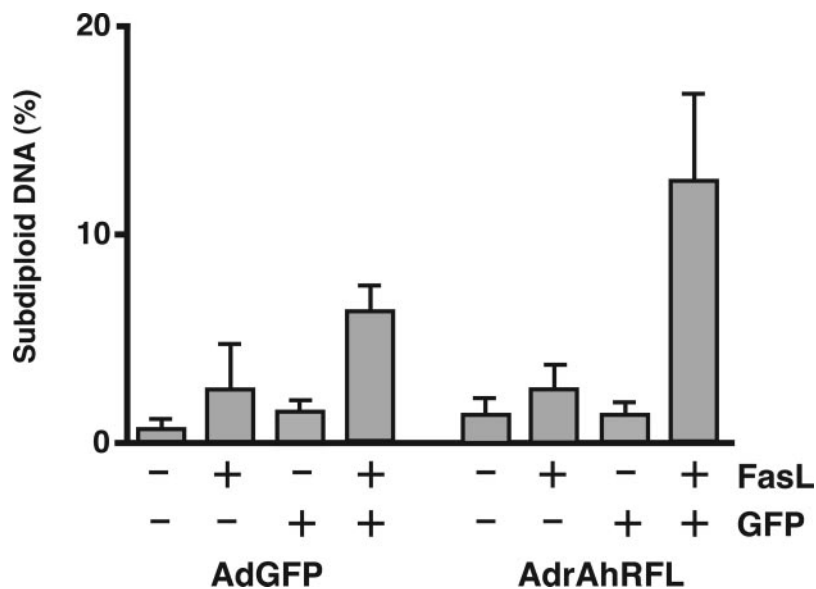
## Discussion

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. Although 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 after 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 proapoptotic and/or antiapoptotic 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 (Ogasawara et al., 1993). In contrast, three of the four AhR knockout mice survived for 24 h, as did the control (saline-treated) mice. Moreover, the single fatality survived much longer (22 h) than any of the wild-type animals. Although we recognize that the reported 50% portosystemic shunt in AhR<sup>-/-</sup> 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- $\mu$ g dose of Jo2, recognizing that as little as 1  $\mu$ g of Jo2 will induce lethal liver apoptosis in some mice (Redondo et al., 1996), whereas 3.75  $\mu$ g killed seven of eight animals (de la Coste et al., 1999) and 5  $\mu$ g was lethal to all of the mice (Takehara et al., 1999). Furthermore, the liver is the primary



**Fig. 6.** Expression of proapoptotic and antiapoptotic Bcl-2 family proteins in BP8 and BP8-WT cells. Total cell lysates (20  $\mu$ g) 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 22-kDa precursor. TfR is included as a loading control.

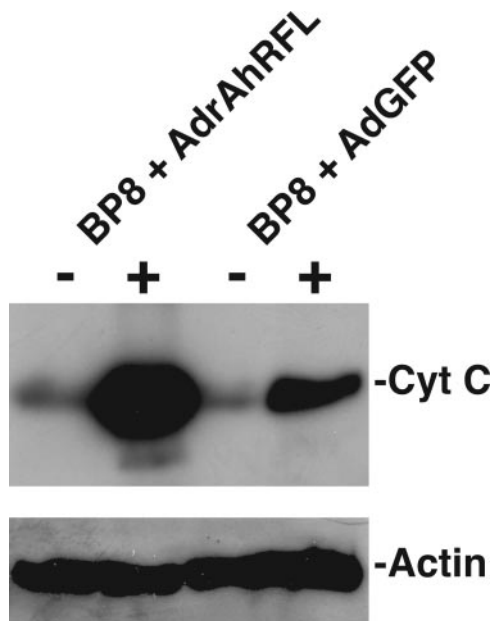


**Fig. 7.** AhR expression in BP8 cells by adenoviral infection promotes FasL induced apoptosis. Subconfluent BP8 cell cultures were infected for 48 h with AdGFP or AdrAhRFL at an m.o.i. of 50 to obtain a population of both infected and uninfected cells in the same culture. Cultures were untreated (-FasL) or treated with 50 ng/ml FasL/6 h (+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  $\pm$  S.E.M. of at least four independent experiments.

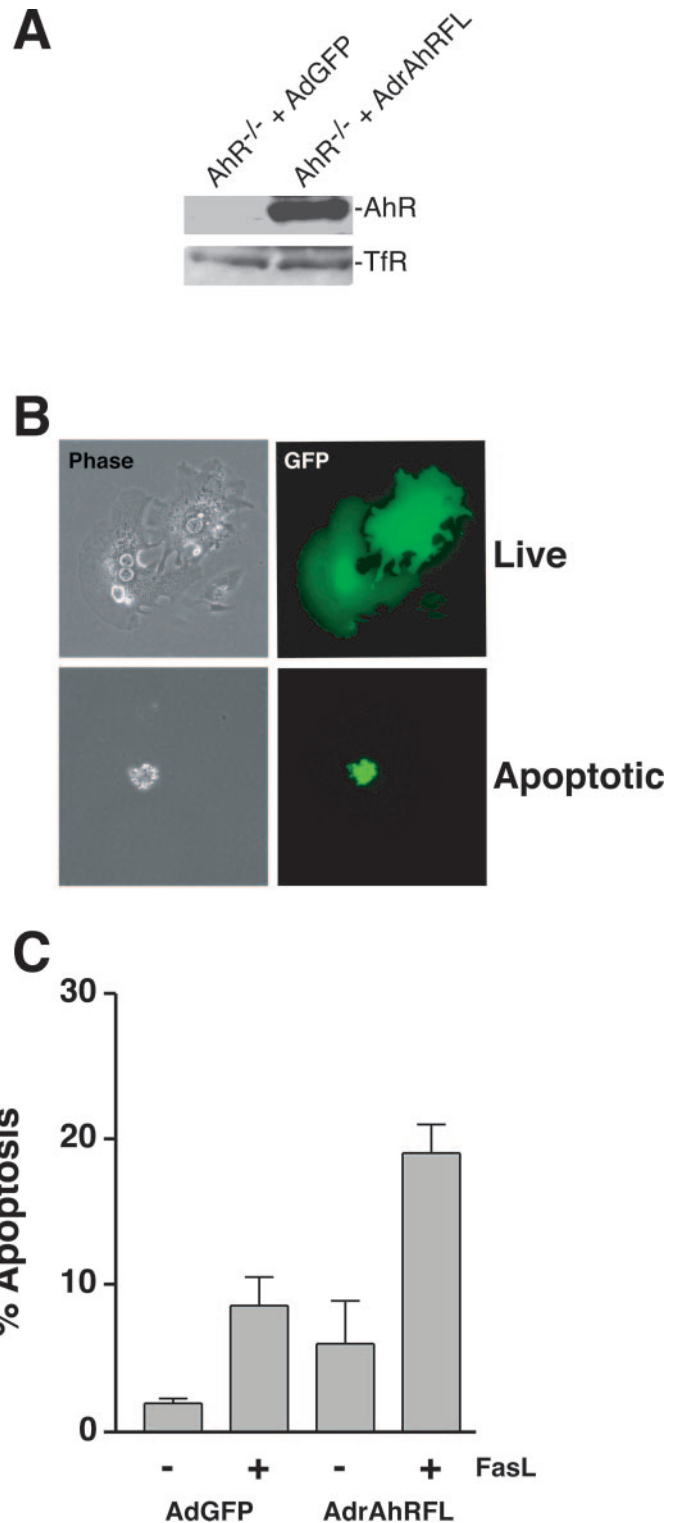


target for Jo2-induced apoptosis, suggesting that the liver in knockout mice will eventually be exposed to the Jo2 antibody despite the portal shunt. This said, however, we cannot absolutely exclude the possibility that AhR<sup>-/-</sup> mice are protected from Jo2-induced apoptotic death caused by the shunt. In this context, it is worth noting that isolating primary hepatocytes from the knockout mice—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<sup>-/-</sup> mice are significant because they support the *in vivo* observations without concerns about the shunt (Fig. 9). 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.

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 in virus-infected primary hepatocytes from AhR<sup>-/-</sup> mice (Fig. 9). The enhanced release of mitochondrial Cyt *c* in AhR-positive cells after FasL stimulation (Figs. 3 and 8) suggests that the proapoptotic 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



**Fig. 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 m.o.i. of 100. Cultures were treated with PBS (–) or 50 ng/ml FasL/4 h (+), and the cytosol (20  $\mu$ g) was analyzed by Western blotting for Cyt *c* and actin (used as a loading control).

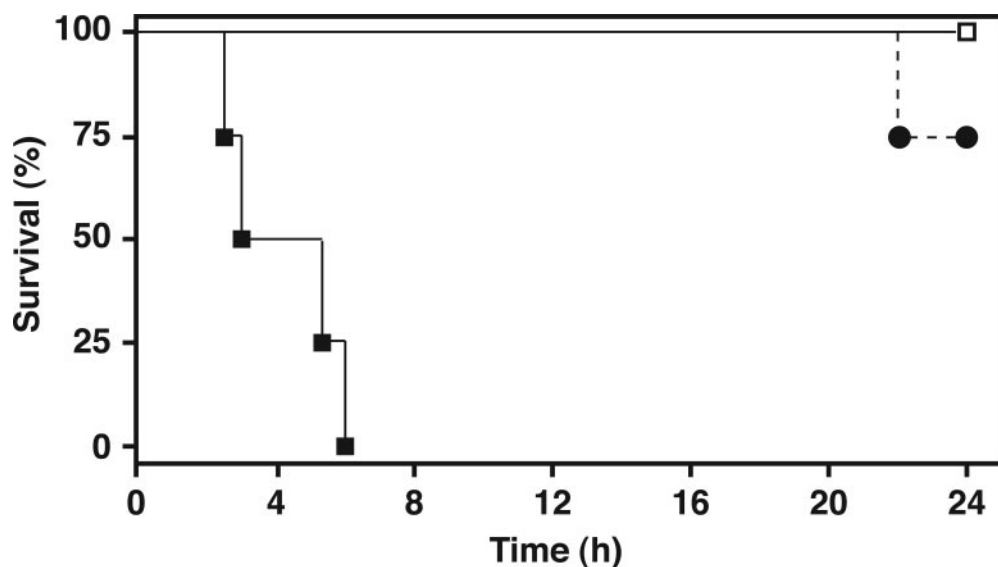


**Fig. 9.** AhR expression in primary hepatocytes from AhR<sup>-/-</sup> mice increases FasL-induced apoptosis. Primary hepatocytes were isolated by collagenase perfusion from AhR<sup>-/-</sup> mice. Cells were infected with AdGFP or AdrAhRFL at a m.o.i. of 100 and maintained in culture for 48 h before treatment with PBS (–) or 50 ng/ml FasL (+) for 6 h. A, AhR expression in AdrAhRFL-infected cells was confirmed by Western blotting on total cell lysates (10  $\mu$ g) 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 represent the mean  $\pm$  S.E.M. from two independent experiments in which at least 200 cells were scored for each condition.

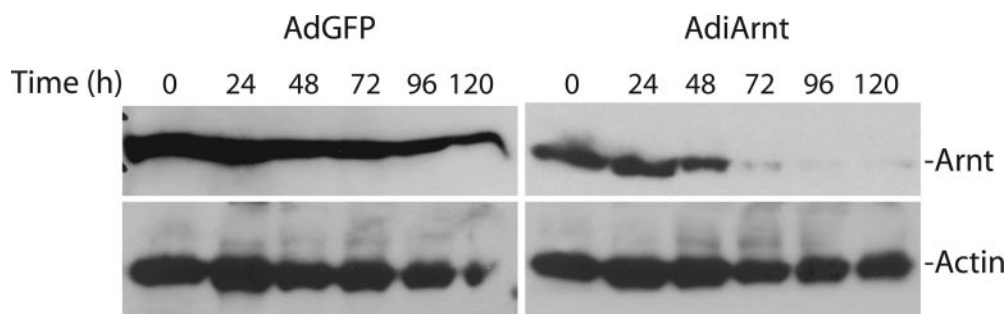


family members (Fig. 6) in BP8 and BP8-WT cells suggests that these are not AhR target genes in liver cells. These measures of protein expression do not address their subcellular distribution or function, however, and it remains a formal possibility that the difference in apoptotic susceptibility is tied to the activity of one or other of these proteins. However, the data show that the apoptotic susceptibility of

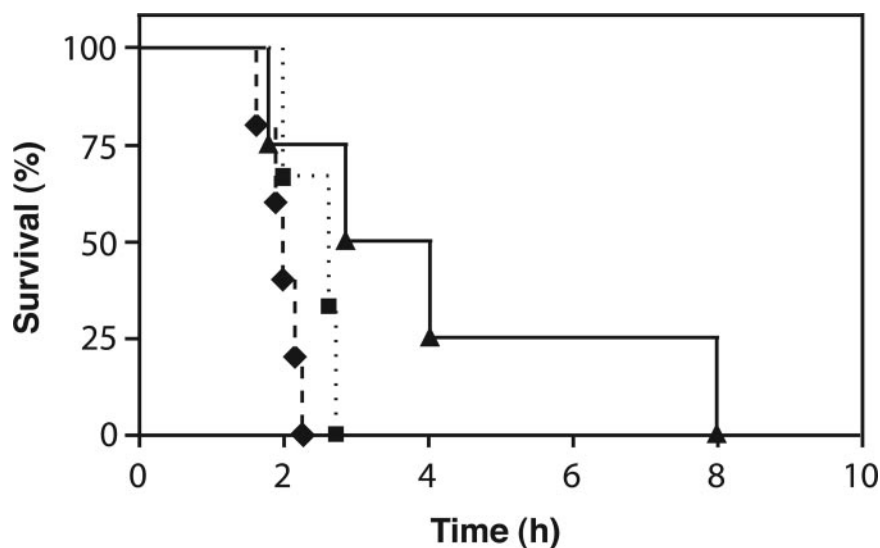
BP8-WT cells is not simply caused by altered expression of Fas or one (or more) of the Bcl-2 family proteins known to participate in Fas-mediated hepatocyte apoptosis. The finding that Bax gene expression is unaffected by AhR expression in the hepatocytes is particularly significant given the recent observation that the Bax gene is a receptor target gene in mouse oocytes in response to polycyclic aromatic hydrocar-



**Fig. 10.** Survival of wild-type and AhR<sup>-/-</sup> mice after treatment with the Jo2 antibody. C57BL/6 wild-type mice (■, *n* = 4) and C57BL/6-Ahr<sup>tmBra</sup> AhR knockout mice (●, *n* = 4) were injected intravenously with 10 μg/head Jo2 antibody in 50 μl of PBS and followed for up to 24 h. Wild-type control mice (□, *n* = 2) were injected with 50 μl of PBS. Percentage survival was plotted against time of death for each animal until 24 h.



**Fig. 11.** Suppressing Arnt protein expression protects mice from Jo2-induced lethality. C57BL/6 mice were injected intraperitoneally with GdCl<sub>3</sub> (10 mg/kg body weight) at 30 and 6 h before adenovirus administration. Adenovirus was administered (10<sup>9</sup> efu in 100 μl of PBS) via tail vein infusion, and 4 days later, mice were injected with 10 μg/head Jo2 via tail vein infusion and monitored for survival. Treatments included GdCl<sub>3</sub> alone (■, *n* = 3), GdCl<sub>3</sub> and AdGFP (◆, *n* = 5), and GdCl<sub>3</sub> and AdiArnt (▲, *n* = 4). The result represents the collective survival data for all mice from two independent experiments.



**Fig. 12.** Adenovirus-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 to 5 days with AdiArnt expressing an 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).

bons (Matikainen et al., 2001). Given that Bax does not seem 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 antiapoptotic proteins Bcl-2 and Bcl-xL can account for the proapoptotic response.

Assessment of caspase-8 and caspase-9 activity in BP8 and BP8-WT cells after 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 downstream 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 after FasL stimulation for 4 h, which heralds the increased incidence of PARP cleavage (Fig. 2) and DNA degradation (Fig. 1) in these cells. Together, these data suggest that AhR function in liver cells affects the Fas pathway downstream of the DISC, but upstream of caspase-9 activation, by a mechanism promoting Cyt c release from the mitochondria. Reiniers and Clift (1999) showed that AhR function in the mouse Hepa-1 cells also enhanced C<sub>2</sub>-ceramide-induced apoptosis by a mechanism not dependent on direct AhR participation. C<sub>2</sub>-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 proapoptotic activity seems to affect mitochondrial Cyt c release, possibly by regulating the expression or function of either pro- or antiapoptotic proteins that control Cyt c release.

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**Address correspondence to:** Dr. Cornelis J. Elferink, Department of Pharmacology and Toxicology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1031. E-mail: coelferi@utmb.edu

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