Distinct response to dioxin in an arylhydrocarbon receptor (AHR)-humanized mouse

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There are large inter- and intraspecies differences in susceptibility to dioxin-induced toxicities. A critical question in risk assessment of dioxin and related compounds is whether humans are sensitive or resistant to their toxicities. The diverse responses of mammals to dioxin are strongly influenced by functional polymorphisms of the arylhydrocarbon receptor (AHR). To characterize responses mediated by the human AHR (hAHR), we generated a mouse possessing hAHR instead of mouse AHR. Responses of these mice to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene were compared with the responses of naturally sensitive (C57BL/6J) and resistant (DBA/2) mice. Mice homozygous for h*AHR* exhibited weaker induction of AHR target genes such as *cyp1a1* and *cyp1a2* than did C57BL/6J ($Ahr^{b\text{-}1/b\text{-}1}$) mice. DBA/2 ($Ahr^{d/d}$) mice were less responsive to induction of *cyp* genes than C57BL/6J mice. hAHR and DBA/2 AHR exhibit similar ligand-binding affinities and homozygous h*AHR* and $Ahr^{d/d}$ mice displayed comparable induction of AHR target genes by 3-methylcholanthrene. However, when TCDD was administered, a greatly diminished response was observed in homozygous h*AHR* mice compared with $Ahr^{d/d}$ mice, indicating that hAHR expressed in mice is functionally less responsive to TCDD than DBA/2 AHR. After maternal exposure to TCDD, homozygous h*AHR* fetuses developed embryonic hydronephrosis, but not cleft palate, whereas fetuses possessing $Ahr^{b\text{-}1}$ or Ahr^{d} developed both anomalies. These results suggest that hAHR may define the specificity of the responses to various AHR ligands. Thus, the hAHR knock-in mouse is a humanized model mouse that may better predict the biological effects of bioaccumulative environmental toxicants like TCDD in humans.

human | C57BL6/J | DBA/2 | CYP1A1

Polycyclic aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons (HAH), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[*a*]pyrene, and polychlorinated biphenyls, are ubiquitous environmental toxicants whose chemical stability and lipophilicity make them highly persistent in the environment and in living organisms. These groups of chemicals cause various toxicological and biological responses, typified by teratogenesis, thymic atrophy, severe epithelial disorders, wasting syndrome, tumor promotion, and induction of xenobiotic-metabolizing enzymes in experimental animals (1, 2). The toxicities of these compounds are mediated by a conserved signaling pathway (1–4) through binding to and activation of the arylhydrocarbon receptor (AHR). AHR activation in turn mediates a transcriptional response for genes regulated by this transcription factor (5–8). Despite strong conservation of this pathway, there are wide inter- and intraspecies differences in the toxicological responses to AHR ligands (9–11). The molecular basis for these species and strain differences appears to relate to polymorphisms in AHR. Factors influencing susceptibility to the toxicity of TCDD have been studied in several animal models. There is a 10-fold difference in susceptibility between the dioxin-sensitive C57BL/6 and the resistant DBA/2 strains of mice that can be explained by polymorphic variations in the ligand-binding domain and in the C-terminal region of the AHR molecule of each strain (9, 12–14). Response to TCDD in the Long–Evans (sensitive) and Han/Wistar rats (resistant) differs by >1,000-fold due to a critical point mutation in the transactivation domain in the AHR of the Han/Wistar rat (15–17).

The effects of TCDD on humans are less well understood, although high incidences of chloracne, teratogenicity, and abortion have been associated with high blood concentrations of dioxin and related compounds in residents of regions where industrial accidents or extensive use of dioxin-containing defoliants have resulted in human exposures (3). Increased levels of dioxin in the body have been reported recently to be associated with abnormal sex ratio of newborns nearly 25 years after the accident in Seveso, Italy (18). Because the AHR primarily mediates the pleiotropic manifestations of dioxin exposure, characterization of the structural and functional properties of the human AHR (hAHR) is critical for understanding the types and magnitudes of human responses to various PAH/HAHs.

To date, *in vitro* characterization of the hAHR has provided ambiguous insights into human sensitivity to dioxin. The dissociation constant (K_d) of hAHR for TCDD was comparable to that of TCDD-resistant DBA/2 AHR (9, 19), suggesting that humans might be resistant to TCDD. By contrast, high homology of the human receptor to the AHR of the guinea pig, which is the most sensitive animal to TCDD, suggests a high responsiveness of humans to the toxin (20). Ligand specificity of hAHR was also examined and compared with those of zebrafish and rainbow trout AHRs using polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners as test ligands. These studies revealed that mono-ortho polychlorinated biphenyls activated hAHR but were not very effective in activating either zebrafish or rainbow trout AHRs (21).

Assessment of human responses *in vivo* to unintended exposures to various PAH/HAHs has been hampered by limited exposure assessments and toxicological follow-up. Observational studies after intentional exposures have not been and should not be conducted. To gain stronger insight into the hazards to human health posed by compounds interacting with the hAHR *in vivo*, we generated a mouse model that harbors the hAHR cDNA instead of the mouse *AhR* gene. This mouse may reveal a humanized susceptibility to chemical toxicities. In response to challenges with 3-methylcholanthrene (3-MC) and TCDD, two prototypical AHR ligands, the hAHR knock-in

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Abbreviations: AHR, aryl hydrocarbon receptor; hAHR, human AHR; h*AHR*, human AHR knock-in allele; Ahr^{d}, DBA/2 *Ahr* allele; $Ahr^{b\text{-}1}$, C57BL/6 *Ahr* allele; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3-MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbons; HAH, halogenated aromatic hydrocarbons; ES, embryonic stem; GD, gestation day.

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mouse displayed a distinct response profile compared with control animals harboring either the C57BL/6 *Ahr* allele ($Ahr^{b\text{-}1}$) (TCDD-sensitive C57BL/6J AHR) or the DBA/2 *Ahr* allele (Ahr^{d}) (TCDD-resistant DBA/2 AHR) in the same C57BL/6J genetic background. Although gene expression responses mediated by hAHR from 3-MC were comparable to that by DBA/2 AHR, the homozygous human AHR knock-in allele (h*AHR*) mouse was the weakest responder to TCDD among the three strains examined. These results suggest that hAHR molecules expressed in mice retain a functional human specificity that can be distinguished from the murine AHR and provide important insights into the toxicological susceptibility of humans to AHR ligands released into the environment.

Materials and Methods

Construction of the hAHR Knock-in Vector. The hAHR knock-in vector was constructed by using 129SV/J mouse *Ahr* genomic clones and hAHR cDNA as described (22). A 2-kb *Bam*HI/*Hph*I fragment containing the 129SV/J *Ahr* promoter was ligated to the hAHR cDNA (9, 22). The *neo* gene cassette was fused to the 3′ end of the hAHR cDNA in a reverse orientation, followed by a 6.5-kb *Hind*III/*Eco*RI fragment of the 129SV/J *Ahr* gene. This construct was ligated to the thymidine kinase cassette on the 5′ end.

Generation of hAHR Knock-in Mice. The knock-in vector was electroporated into E14 embryonic stem (ES) cells (23). A pair of primers (sense, GTATGCATTACCATGCTCCCATTCTGCTGG; antisense, ACATCTTGTGGGAAAGGCAGCAGGCTAGCC) was used for PCR screening. After confirmation by Southern blot analysis, positive clones were injected into blastocysts. Heterozygous hAHR knock-in mice were backcrossed into a C57BL/6J background up to the seventh generation and interbred to yield heterozygous and homozygous h*AHR* and wild-type $Ahr^{b\text{-}1/b\text{-}1}$ mice. The genotype of each pup was determined by PCR, with a common sense primer; 5′-ATGAGCAGCGGCGCCAACAT-3′, an antisense primer for endogenous *Ahr* allele; 5′-GCTAGACGGCACTAGGTAGG-3′, and an antisense primer for targeted allele; 5′-CAGGTAACTGACGCTGAGCC-3′. PCR amplification was carried out for 30 cycles under the following conditions; 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec.

Chemicals and Animals. TCDD (99.5% pure) and 3-MC were purchased from Cambridge Isotope Laboratories (Andover, MA) and Wako Pure Chemical (Osaka), respectively. D2N-*Ahrd* mice and inbred C57BL6/J mice were procured from The Jackson Laboratory. *Ahr*-null mutant mice used in this study were generated by Y.F.-K (22).

RNA Blotting Analyses. We isolated total RNA by using ISOGEN (Nippon Gene, Tokyo) and purified polyA RNA by using an Oligotex-MAG mRNA purification kit (Takara Biotechnology, Tokyo). For detection of Ahr mRNA, 5 μg of polyA RNA per lane was applied, and a portion of mouse $Ahr^{b\text{-}1}$ cDNA (*Bpu*1102I-*Kpn*I; 734-bp) encoding the PAS domain was used for a probe. This nucleotide sequence is conserved with 83% homology to the corresponding hAHR cDNA (12, 24). To examine the inducibility of *CYP1A1* and *CYP1A2,* 6-week-old littermates ($Ahr^{b\text{-}1/b\text{-}1}$ and homozygous h*AHR*) and D2N-*Ahrd* ($Ahr^{d/d}$) mice were given a single i.p. injection of 80 mg/kg 3-MC or 100 μg/kg TCDD. Mice were killed by cervical dislocation 24 h after injection. Ten micrograms of total RNA per lane was hybridized with the appropriate mouse cDNA probes (25).

RT-PCR Analyses of hAHR and Murine Ahr mRNA Expression in Embryos. Total RNA was isolated from palate and kidney of gestation day (GD)18.5 fetus by using ISOGEN. One microgram of the total RNA was reverse-transcribed into cDNA with Superscript-II reverse transcriptase (Life Technologies, Gaithersburg, MD) and random hexamers at 42°C for 50 min. The resulting cDNAs were subjected to 30 cycles of PCR by using the specific primers for the gene for the h*AHR* (5′ primer, 5′-GTAAGTCTCCCTTCATACC-3′; 3′ primer, 5′-AGGCACGAATTGGTTAGAG-3′), mouse *Ahr* (5′ primer, 5′-CTTTGCTGAACTCGGCTTGC-3′; 3′ primer, 5′-TTGCTGGGGGCACACCATCT-3′) and GAPDH (5′ primer, 5′-CCCCTTCATTGACCTCAACTACATGG-3′; 3′ primer, 5′-GCCTGCTTCACCACCTTCTTGATGTC-3′). The reaction was performed under the following conditions: 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec.

Immunohistochemical Analysis of hAHR Expression. Immunohistochemical analysis was performed as described (26). Lungs were fixed in 0.1 M phosphate buffer containing 4% paraformaldehyde for 24 h and embedded in paraffin. Sections were incubated with anti-AHR antibody in 1:200 dilution, which reacts with both human and mouse AHR (N-19; Santa Cruz Biotechnology). AHR immunoreactivity was visualized with the avidin-biotin-peroxidase system (Vector Laboratories).

TCDD Treatment and Evaluation of Teratogenesis. TCDD treatment was performed as described (22). On GD12.5, pregnant mice were given TCDD by i.p. administration at a dose of 40 μg/kg body weight (27). On GD18.5, the fetuses were taken out and fixed in 4% paraformaldehyde. The palatal structure was examined by cutting between the upper and lower jaws. The kidneys were sliced longitudinally and stained with hematoxylin/eosin. The presence and severity of hydronephrosis in each kidney was examined under a microscope as previously described (28) by using severity scores ranging from 0 to 3+ (0, normal kidney; 1+, slight decrease in length of papilla; 2+, marked decrease in length of papilla with some loss of renal parenchyma; 3+, complete absence of papilla, shell of kidney remaining with only a small amount of renal parenchyma). For statistical analysis, pairwise comparisons were made by Mann–Whitney *U* test, by using StatView for Macintosh version 5.0 (SAS Institute, Cary, NC).

Results

Replacement of the Mouse *Ahr* Gene with hAHR cDNA. We hypothesize that the specific functional characteristics of the hAHR molecule form the principal basis for the pattern of human responses to xenobiotics that interact with the AHR. To characterize responses mediated by hAHR, we generated a mouse possessing hAHR instead of murine AHR. hAHR cDNA was introduced into the mouse *Ahr* locus by homologous recombination, thereby disrupting the mouse *Ahr* gene (Fig. 1*A*). The cDNA was recombined so that hAHR is expressed under the control of the endogenous mouse *Ahr* promoter. Sixteen independent G418-resistant ES clones were obtained of 240 by PCR screening, and seven clones were further confirmed as correctly targeted ES cells by genomic DNA blot analysis. *Eco*RI-digested genomic DNA from the three representative positive clones (nos. 14, 25, and 58) revealed 11.0- and 6.2-kb fragments derived from the intact and targeted alleles, respectively, when hybridized with the 5′-external probe (Fig. 1*B*).

These three clones harboring h*AHR* were used for the generation of chimeric offspring. The male chimeras were mated with C57BL/6J females to obtain heterozygotes of the h*AHR* allele. They were subsequently bred into a C57BL/6J genetic background through the seventh generation, and the backcrossed heterozygous animals were interbred to yield hAHR homozygous mutant mice. The transmission of the targeted allele to the offspring was confirmed by genomic DNA blot analysis, and the genotype was determined by PCR by using tail DNA as a template (Fig. 1 *C* and *D*). Of 124 offspring obtained

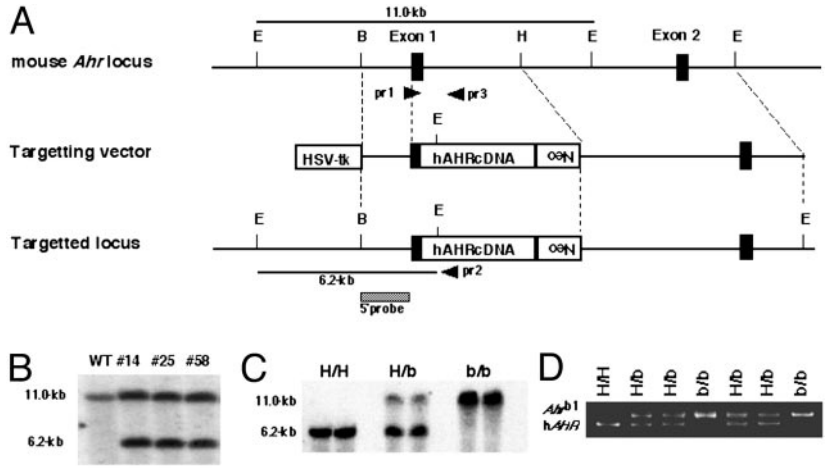

Fig. 1. Generation of the hAHR knock-in mouse. (*A*) Strategy for hAHR cDNA knock-in by homologous recombination. E, H, and B are restriction sites for *Eco*RI, *Hind*III, and *Bam*HI, respectively. Neo indicates the neomycin-resistance gene, and HSV-tk is the thymidine kinase gene under control of the herpes simplex virus promoter. The 5′-genomic probe used for DNA blot analysis is indicated by the hatched box. The positions of wild-type (pr 3) and mutant allele-specific (pr 2) primers and the common primer (pr 1) used in the genotyping PCR are indicated by arrowheads. The *Eco*RI restriction fragments detected with the 5′-genomic probe in the wild-type and targeted allele are denoted by horizontal bars. (*B*) DNA blot analyses of three recombinant ES clones. Genomic DNA was prepared from the ES clones (nos. 14, 25, and 58), and aliquots (10 μg) were digested by *Eco*RI. *Eco*RI digestion generated 11.0- and 6.2-kb bands for the wild-type and targeted alleles, respectively, by using the 5′-genomic probe. (*C*) Genotyping of the *Ahr* gene by DNA blot analysis. Genomic DNA was extracted from the tails of heterozygous and homozygous h*AHR* mice and wild-type $Ahr^{b\text{-}1/b\text{-}1}$ mice and digested by *Eco*RI for DNA blot analysis. (*D*) Genotyping of littermates from the intercrosses of heterozygotes. PCR fragments of wild-type amplified with pr1 and pr3 ($Ahr^{b\text{-}1}$; 280 bp) and mutant allele with pr1 and pr2 (h*AHR*; 240 bp) as depicted in *A*. H/H, H/b, and b/b indicate homozygous and heterozygous h*AHR* mice and wild type ($Ahr^{b\text{-}1/b\text{-}1}$), respectively.

from heterozygous matings, wild-type ($Ahr^{b\text{-}1/b\text{-}1}$), heterozygous, and homozygous h*AHR* mutant mice numbered 29, 71, and 24, respectively, conforming to the expected Mendelian inheritance ratio. Homozygous h*AHR* mice were viable, and no abnormalities were observed.

Expression of hAHR in hAHR Knock-in Mice. The expression of hAHR and mouse Ahr mRNAs was examined by RNA blot analysis by using polyA RNAs isolated from major AHR-expressing organs including liver, lung, kidney, intestine, and thymus (Fig. 2*A*). A cDNA fragment encoding the PAS domain of C57BL/6 AHR, which shows 83% homology with the corresponding human molecule, was used as a common probe for detecting both mouse Ahr and hAHR mRNAs. The larger band detected in heterozygous h*AHR* mice and wild-type $Ahr^{b\text{-}1/b\text{-}1}$ mice corresponds to the 5.4-kb transcript derived from the endogenous $Ahr^{b\text{-}1}$ gene, and the shorter 5.0-kb transcript observed in heterozygous and homozygous h*AHR* is derived from the hAHR knock-in allele. This result establishes that, whereas the homozygous h*AHR* mouse lacks mRNA for murine *Ahr*, it expresses mRNA for h*AHR*. Further, the level of expression of hAHR mRNA is comparable to that of endogenous murine Ahr mRNA in the other strains.

The embryonic expressions of mouse Ahr and hAHR mRNAs were examined by RT-PCR at the stage of GD18.5. As observed in the RNA blot analysis of adult tissues, the hAHR mRNA was expressed in the embryonic palate and kidney of homozygous and heterozygous h*AHR* mice. The abundance was comparable with that of the mouse Ahr mRNA expressed in $Ahr^{b\text{-}1/b\text{-}1}$ and heterozygous h*AHR* mice (Fig. 2*B*). These results demonstrate that hAHR mRNA is transcribed under the control of the mouse *Ahr* promoter in both adult and embryonic hAHR knock-in mice.

To ascertain that hAHR protein is expressed from the knock-in allele, immunohistochemical analysis was performed on lung sections obtained from hAHR knock-in homozygous mouse and the *Ahr*-null mutant (22). Intense signals were detected in the alveolar epithelial cells of hAHR knock-in animals (Fig. 2*C*). The signal intensity of *Ahr*-null mutant lung (Fig. 2*D*) was as faint as the hAHR knock-in lung without the antibody (data not shown). Thus, hAHR protein is expressed from the knock-in allele.

The hAHR Knock-in Mouse Displays a Distinct Induction Profile of AHR Target Genes to Different AHR Ligands. The response of the hAHR knock-in mouse to two prototypical AHR ligands, 3-MC and TCDD, was examined. To characterize the distinct properties, if any, of the hAHR, two strains of control mice were used for the analysis. One strain is a wild-type mouse in the C57BL/6J genetic background, which possesses AHR with high affinity for TCDD. The other strain is a congenic mouse, D2N-*Ahrd*, possessing AHR with low affinity (from DBA/2 mouse) in the C57BL6J genetic background. Because the hAHR knock-in mouse was backcrossed into C57BL/6J, these two strains of mouse enabled us to compare the characteristics of hAHR to those of C57BL/6J and DBA/2 AHR in the same genetic background.

Robust expression of the *CYP1A1* and *CYP1A2* genes was observed in the liver of $Ahr^{b\text{-}1/b\text{-}1}$ mice after administration of 3-MC, whereas the magnitudes of induction in homozygous h*AHR* and $Ahr^{d/d}$ mice were much weaker and comparable to each other (Fig. 3*A*). The relative mean band intensities for *CYP1A1* were 1.0 and 0.9 and were 1.0 and 1.1 for *CYP1A2* in homozygous h*AHR* and $Ahr^{d/d}$ mice, respectively. After treatment with TCDD, the induction of the two genes was strongest in $Ahr^{b\text{-}1/b\text{-}1}$ mice, intermediate in $Ahr^{d/d}$ mice, and weakest in homozygous h*AHR* mice (Fig. 3*B*). The fold inductions in homozygous h*AHR*, $Ahr^{d/d}$, and $Ahr^{b\text{-}1/b\text{-}1}$ mice were 1.0, 4.9, and 14.6 for *CYP1A1*, and 1.0, 5.7, and 8.4 for *CYP1A2*, respectively.

When the responses of $Ahr^{b\text{-}1/b\text{-}1}$ and $Ahr^{d/d}$ mice were compared, the *CYP1A1* expression levels were higher in $Ahr^{b\text{-}1/b\text{-}1}$ than in $Ahr^{d/d}$ mice, which is consistent with previous reports (9, 12, 13). It is noteworthy that the responsiveness of homozygous h*AHR* mice to 3-MC was almost comparable to that of $Ahr^{d/d}$ mice, whereas the responsiveness to TCDD was much weaker. The differential response between $Ahr^{d/d}$ and homozygous h*AHR* mice was unexpected, because a previous study indicated

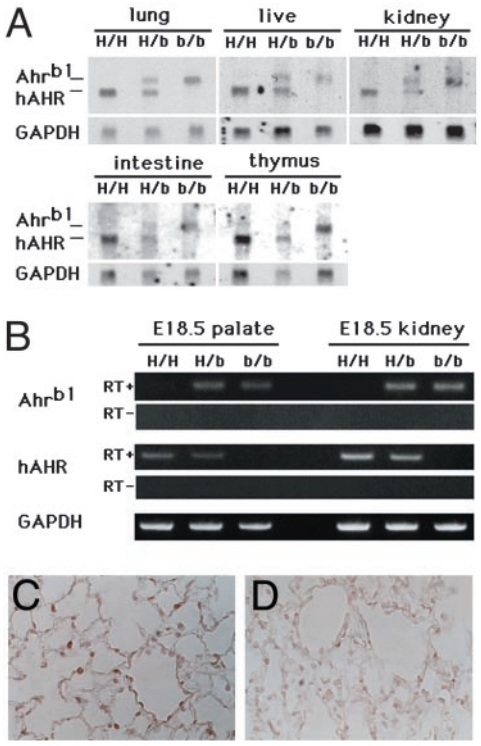

Fig. 2. Expression of hAHR in multiple tissues of the hAHR knock-in mouse. (*A*) RNA blot analysis of polyA RNA (5 μg/lane) extracted from five representative organs of homozygous and heterozygous h*AHR* mice and $Ahr^{b\text{-}1/b\text{-}1}$ mice. Human and mouse *Ahr* transcripts (hAHR and $Ahr^{b\text{-}1}$, respectively) are indicated (*Left*). The same membrane was rehybridized with ^{32}P-labeled cDNA of mouse *GAPDH*. H/H, H/b, and b/b are described in the Fig. 1 legend. (*B*) RT-PCR analyses of hAHR and murine Ahr mRNA expression in kidney and palate of GD18.5 fetuses. The reverse transcription was conducted either in the presence (+) or absence (−) of reverse transcriptase. PCR products representing the transcripts derived either from h*AHR* or $Ahr^{b\text{-}1}$ are indicated on the left. (*C* and *D*) Immunohistochemical analysis of hAHR protein in the lung of a homozygous h*AHR* mouse. Immunoreactivity of AHR protein was observed in the alveolar epithelial cells of homozygous h*AHR* lung (*C*), whereas no immunoreactivity was observed in the lung of $Ahr^{-/-}$ mouse (*D*). Original magnifications, ×400 (*C* and *D*).

that hAHR and DBA/2 AHR exhibit similar dissociation constants for TCDD binding as measured *in vitro* (9, 19). This result suggests that ligand binding does not fully define the integrated function of hAHR.

hAHR Knock-in Mouse Is Relatively Resistant to TCDD-Induced Teratogenicity. The responses to TCDD mediated by hAHR are weaker than that by DBA/2 and C57BL/6 AHR when measured

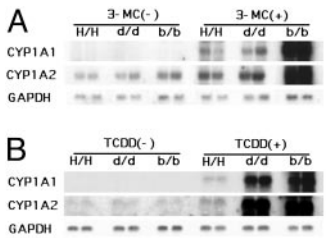

Fig. 3. Inducible expression of AHR target genes. Northern blot analysis of AHR-regulated *CYP1A1* and *CYP1A2* was performed. Six-week-old homozygous h*AHR*, $Ahr^{d/d}$, and $Ahr^{b\text{-}1/b\text{-}1}$ mice were treated with 80 mg/kg 3-MC (*A*) or 100 μg/kg TCDD (*B*). Total hepatic RNA was isolated 24 h after treatment and subjected to Northern analysis (10 μg/lane). Equal loading was confirmed by the abundance of *GAPDH* transcripts.

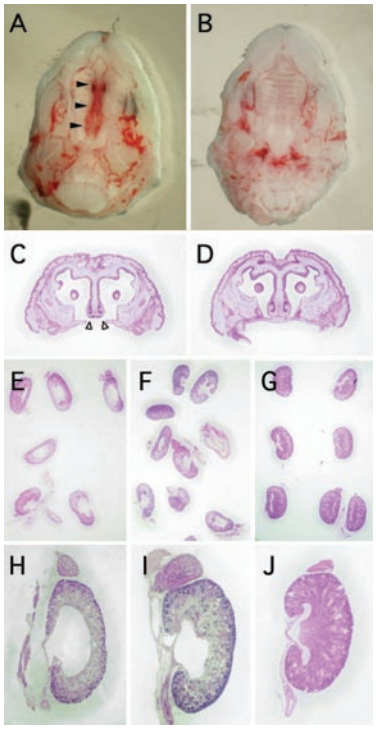

Fig. 4. Fetal teratogenesis after maternal administration of TCDD. (*A* and *C*) Cleft palate in an $Ahr^{b\text{-}1/b\text{-}1}$ fetus is shown. Filled arrowheads in *A* and open arrowheads in *C* indicate the failure of palatine shelves to fuse. Note that homozygous h*AHR* fetuses showed no cleft palates after TCDD treatment (*B* and *D*). (*E*, *F*, *H*, and *I*) Fetal hydronephrosis induced by TCDD. $Ahr^{b\text{-}1/b\text{-}1}$ (*E* and *H*) and homozygous h*AHR* (*F* and *I*) fetuses are shown. (*G* and *J*) Unaffected kidneys from untreated $Ahr^{b\text{-}1/b\text{-}1}$ fetuses are shown.

as inducibility of *CYP1A* family genes. Teratogenicity is a more integrated and complex toxicological manifestation of TCDD action. The most prominent teratogenic effects of TCDD on mouse fetus are cleft palate and hydronephrosis, both of which depend completely on AHR expression (29). The frequency and severity of these teratogenic effects of TCDD were examined in hAHR knock-in fetuses. Homozygous hAHR knock-in females were mated with males of the same genotype and given a single i.p. dose of 40 μg of TCDD per kg of body weight at GD12.5. $Ahr^{b\text{-}1/b\text{-}1}$ and $Ahr^{d/d}$ females were treated in the same way as controls. All dams were weighed to monitor the normal continuation of the pregnancy and killed at GD18.5 to remove fetuses for examination of cleft palate and hydronephrosis.

As reported previously, cleft palate was observed in 100% of the wild-type $Ahr^{b\text{-}1/b\text{-}1}$ fetuses exposed to TCDD (Fig. 4 *A* and *C* and Table 1) (22). By contrast, none of the treated homozygous h*AHR* fetuses showed abnormal palatogenesis (Fig. 4 *B* and *D* and Table 1). An intermediate frequency (30%) of cleft palate was observed in the $Ahr^{d/d}$ fetuses. Differences in the severity of cleft palate were not apparent in any of the symptomatic fetuses of any genotype. This anomaly was most frequent in $Ahr^{b\text{-}1/b\text{-}1}$, intermediate in $Ahr^{d/d}$, and least frequent in homozygous h*AHR* mice, in accordance with the transcriptional inducibility of AHR target genes, which was strongest in $Ahr^{b\text{-}1/b\text{-}1}$, intermediate in $Ahr^{d/d}$, and weakest in homozygous h*AHR* mice. Thus, a strong correlation between the incidence of cleft palate in each strain and the intrinsic transcriptional activity of their respective AHR molecules was observed.

Hydronephrosis, another teratogenic effect of TCDD, is characterized by a dilated renal pelvis. The severity of this anomaly in the fetal kidney was scored from 0 (normal) to 3 (severest)

Table 1. Incidence of anomalies caused by TCDD in homozygous h*AHR*, *Ahr*$^{b\text{-}1/b\text{-}1}$, and *Ahr*$^{d/d}$ fetuses

Genotype of fetuses	TCDD dose, μg/kg	Dams examined, *n*	Fetuses examined, *n*	Fetuses with: Cleft palate *n*	Cleft palate %*	Hydronephrosis *n*	Hydronephrosis %*	Hydronephrosis Severity: 0–3.0†
Ahr$^{b\text{-}1/b\text{-}1}$	0	2	13	0	0	2	12.5	0.19 ± 0.10‡
Ahr$^{b\text{-}1/b\text{-}1}$	40	5	29	29	100	26	89.7	2.54 ± 0.14§
Ahr$^{d/d}$	0	2	15	0	0	2	13.3	0.20 ± 0.10‡
Ahr$^{d/d}$	40	5	30	9	30	25	81.7	1.98 ± 0.14§
Homo-h*AHR*	0	2	16	0	0	1	6.3	0.03 ± 0.03‡
Homo-h*AHR*	40	5	37	0	0	30	81.1	1.19 ± 0.01§

*Percentage of fetuses with each anomaly of all fetuses examined.
†The criteria for severity scores are described in *Materials and Methods.* Data are expressed as mean ± SE.
‡Significant difference between TCDD-treated and -untreated fetuses of each genotype ($P < 0.0001$).
§Significant difference between TCDD-treated homozygous h*AHR* fetuses and *Ahr*$^{b\text{-}1/b\text{-}1}$ or *Ahr*$^{d/d}$ fetuses ($P < 0.0001$).

according to criteria described previously (28). When kidneys scored at 1, 2, or 3 were counted as hydronephrotic, 89.7% of the *Ahr*$^{b\text{-}1/b\text{-}1}$ offspring suffered from this teratogenic outcome after TCDD treatment (Fig. 4 *E* and *H*, and Table 1 for TCDD-treated animals; Fig. 4 *G* and *J*, and Table 1 for untreated animals). A similar incidence was observed in a previous study (22). *Ahr*$^{d/d}$ and homozygous h*AHR* fetuses also displayed this teratogenic effect with incidences of 81.7% and 81.1%, respectively (Fig. 4 *F* and *I*, and Table 1). Thus, there is no substantial difference in the incidence of hydronephrosis among the mice expressing the three distinct *Ahr* (h*AHR*) genes. When severity score values were compared among the TCDD-treated fetuses, they averaged 2.54, 1.98, and 1.19 for the *Ahr*$^{b\text{-}1/b\text{-}1}$, *Ahr*$^{d/d}$ and homozygous h*AHR* genotypes, respectively (Table 1). Therefore, hydronephrosis observed in the homozygous h*AHR* fetuses was significantly less severe compared with that in either *Ahr*$^{b\text{-}1/b\text{-}1}$ or *Ahr*$^{d/d}$ fetuses. Nonetheless, the average score of TCDD-treated homozygous h*AHR* fetuses (1.19) was still significantly higher than that of untreated homozygous h*AHR* fetuses (0.03), clearly demonstrating that the TCDD-activated hAHR mediates renal teratogenesis in mice. Although the magnitude of *CYP* gene induction is dramatically different depending on the *Ahr* genotype, the incidence of hydronephrosis is surprisingly comparable among the three strains. These results revealed that differences between human and murine AHR allowed for the emergence of discrete biological effects; e.g., hydronephrosis, but not cleft palate in homozygous h*AHR* mice.

To exclude the possibility that maternal factors affect the teratogenic manifestations on the fetuses, heterozygous h*AHR* parents were used to obtain homozygous h*AHR* and *Ahr*$^{b\text{-}1/b\text{-}1}$ fetuses. Heterozygous mothers were treated with TCDD as described above, and fetuses were examined for both cleft palate and hydronephrosis. As described in Table 2, the incidence of cleft palate was 100% and 0% in *Ahr*$^{b\text{-}1/b\text{-}1}$ and homozygous h*AHR* fetuses, respectively, which is identical to the results presented in Table 1. The incidence and severity (mean score) of hydronephrosis were 100% and 2.47 for *Ahr*$^{b\text{-}1/b\text{-}1}$ and 66.6% and 1.17 for homozygous h*AHR* fetuses, respectively. Again, a more moderate effect in the homozygous h*AHR* fetuses is suggested, the severity difference being statistically significant. Therefore, we conclude that the TCDD-induced teratogenic effects are independent of maternal genotypes, and that fetal AHR activity is critical for determining the outcomes.

Discussion

One of the central issues in the uncertainty surrounding risk assessments for TCDD and its structural analogs is whether humans are relatively sensitive or resistant to the toxicities of this class of compounds. Because the pleiotropic adverse effects induced by these toxins involve multiple processes, the human response is generated by the summation and integration of the properties inherent to the human components, including expression level, ligand-binding affinity, and transcriptional activity of the AHR, as well as the variety, function and activity of the AHR target genes. Through numerous preceding studies, the primary structure of the AHR protein has been regarded as one of the most critical factors determining the susceptibility and specificity of responses of animals to various PAH/HAHs including dioxin. On the basis of several observations *in vitro*, polymorphic variation in the *Ahr* gene is considered the primary basis for differences in sensitivity to TCDD among strains of mice (9–11). In this study, we attempted to establish an *in vivo* system to evaluate the specific function of the hAHR protein to better evaluate its role in determining possible patterns of human responses to PAH/HAHs.

For this purpose, we adopted a knock-in strategy to introduce hAHR cDNA into the mouse *Ahr* genomic locus by homologous recombination. This strategy offers an obvious advantage compared with a transgenic method, because the introduced sequence is transcribed under the same regulatory mechanisms of the replaced gene (30). As desired, expression levels of the h*AHR* transcript were almost the same with those of endogenous mouse

Table 2. Incidence of anomalies caused by TCDD in fetuses from heterozygous h*AHR* parents

Genotype of fetuses	TCDD dose, μg/kg	Dams examined, *n*	Fetuses examined, *n*	Fetuses with: Cleft palate *n*	Cleft palate %*	Hydronephrosis *n*	Hydronephrosis %*	Hydronephrosis Severity: 0–3.0
Ahr$^{b\text{-}1/b\text{-}1}$	40		9	9	100	9	100.0	2.47 ± 0.14†
Hetero-h*AHR*	40	7	25	12	48	22	88.0	2.46 ± 0.13
Homo-h*AHR*	40		12	0	0	8	66.6	1.17 ± 0.01†

*Percentage of fetuses with each anomaly out of all fetuses examined.
†Significant difference between homozygous h*AHR* and *Ahr*$^{b\text{-}1/b\text{-}1}$ fetuses ($P < 0.0001$).

Ahr mRNA in multiple AHR-expressing tissues of adult mice and GD18.5 embryos. The hAHR protein was detected by immunostaining in the lungs of homozygous h*AHR* mice.

A possible explanation of the relative resistance of the hAHR knock-in mouse to TCDD lies in the qualitative difference between the human and mouse AHR molecules. Assuming that the abundance of the hAHR protein is the same as that of the endogenous mouse AHR, our results imply that the hAHR-mediated response to TCDD *in vivo* is much lower than that of DBA/2 AHR, although previous reports showed that their affinities to TCDD, as measured *in vitro*, are almost the same (9, 19). Alignment of the primary amino acid sequences of the two molecules indicates the considerable divergence in the C-terminal regions (9) and the deletion analysis differently localized the transcriptional activity within the regions (31). Such structural diversity of the C-terminal region might lead to species-specific interaction behaviors with transcriptional cofactors. TCDD-activated hAHR may not recruit coactivators as efficiently as the DBA/2 counterpart. One possibility must be noted that the incompatibility between TCDD-activated hAHR and the mouse coactivators may cause the reduced response of hAHR knock-in mouse to TCDD.

hAHR was not detectable by immunoblot analysis with the current antiserum, and its abundance relative to the constitutive level of mouse AHR protein could not be determined. Considering this lack of quantitative information, limited protein accumulation might account for the attenuated responsiveness of hAHR knock-in mice to TCDD. hAHR may have an intrinsically shorter life than mouse AHR at physiological expression levels *in vivo*.

The susceptibility of embryonic kidneys of homozygous h*AHR* mice to the teratogenic effects of TCDD is noteworthy. The pathogenesis of this renal lesion induced by TCDD involves hyperplasia of the ureteric epithelium, resulting in an occlusion of the ureter and subsequent hydronephrosis (32). Adverse effects on the kidney and urinary tract have also been reported in humans exposed to TCDD (33). However, studies in Ben Tre Province in Vietnam, where defoliant containing dioxin was sprayed extensively, revealed little increase in the prevalence of cleft lip and/or palate compared with that observed in Japan (34), suggesting that hAHR is less potent to mediate the manifestation of cleft palate, and that a higher dose might be required for it. Consistent with these human reports, our analysis showed that hAHR, although expressed in mice, mediated the development of hydronephrosis induced by TCDD, but not cleft palate at our experimental dose. Thus, the knock-in animal seems to mimic some aspects of the human responses to PAH/HAHs.

An intriguing utilization of our knock-in mouse strategy would be as an *in vivo* system for the qualitative and quantitative assessment of possible human responses to various PAH/HAHs. In this study, D2N-*Ahrd* mice responded more strongly to TCDD than to 3-MC, whereas the hAHR knock-in mice responded almost equally to these two compounds. These results clearly show that the relative efficacy profiles, examined by TCDD and 3-MC, are different between D2N-*Ahrd* and our hAHR knock-in mouse. Therefore the efficacy profile specific to hAHR can be displayed by analyses of the responses of the hAHR knock-in mouse to an array of PAH/HAHs. Because environmentally relevant levels of exposure to dioxin and related compounds have garnered much concern in terms of their possible effects on reproductive, neurobehavioral, and immunological functions of humans, our hAHR knock-in mouse will serve as a humanized model mouse, exhibiting the human-specific responses to PAH/HAH congeners. This mouse should help define the range of biological and toxicological effects that could be expected to affect humans and thereby reduce some uncertainty in risk assessments of these persistent environmental contaminants.

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