A Hypomorphic Allele of Aryl Hydrocarbon Receptor-Associated Protein-9 Produces a Phenocopy of the Ahr-Null Mouse

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ABSTRACT

The aryl hydrocarbon receptor-associated protein-9 (ARA9) is a chaperone of the aryl hydrocarbon receptor (AHR). The AHR has been shown to play a late developmental role in the normal closure of a fetal hepatovascular shunt known as the ductus venosus (DV). Given that Ara9-null mice display early embryonic lethality, we generated a hypomorphic Ara9 allele (designated Ara9\(^{-}\)\(^{\text{fxneo}}\)) that displays reduced Ara9 protein expression. In an effort to demonstrate the role of ARA9 protein in AHR-mediated DV closure, we used combinations of Ara9 wild-type [Ara9\(^{+/+}\)], null [Ara9\(^{-/-}\)], and hypomorphic [Ara9\(^{\text{fxneo/fxneo}}\)] alleles to produce mice with a graded expression of the ARA9 protein. Liver perfusion studies demonstrated that although none of the Ara9\(^{+/+}\) mice displayed a patent DV, the shunt was observed in 10% of the Ara9\(^{+/\text{fxneo}}\) mice, 55% of the Ara9\(^{+/+}\) mice, and 83% of the Ara9\(^{\text{fxneo/fxneo}}\) mice. That expression level of ARA9 correlates with the frequency of a phenocopy of the Ahr-null allele supports the conclusion that the ARA9 protein is essential for AHR signaling during development.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor best known for mediating the adaptive and toxic responses to polycyclic aromatic hydrocarbons and halogenated dibenzop-dioxins (Poland, 1982; Eisen et al., 1983). Once ligands such as these bind the receptor, the complex translocates to the nucleus in which the AHR dissociates and dimerizes with its transcriptional partner, the aryl hydrocarbon receptor nuclear translocator (ARNT). In the nucleus, the AHR/ARNT dimer binds to cognate “dioxin-responsive elements” in the enhancer regions of responsive genes, resulting in the transcriptional up-regulation of those genes (Reyes et al., 1992; Henry and Gasiewicz, 1993; Hord and Perdew, 1994). The effects mediated by this pathway include the adaptive up-regulation of certain cytochromes P450, as well as toxic endpoints that include thymic involution, hepatocellular damage, and cleft palate (Poland and Knutson, 1982).

It has been shown recently that the AHR also plays an important role in hepatovascular development (Lahvis et al., 2005). In the developing embryo, the ductus venosus (DV) serves as a shunt that connects the umbilical cord blood with the portal vein and the inferior vena cava (IVC). AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; IVC, inferior vena cava; ARA9, aryl hydrocarbon receptor-associated protein-9; bp, base pair(s); kb, kilobase(s); TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.
Our use of Ara9 hypomorphic models was based upon two predictions. First, we hypothesized that mice hypomorphic for Ara9 could be developed that would express enough protein to alleviate the developmental block caused by the essential role of the protein in early cardiac development. Second, if Ara9 was essential for AHR developmental signaling, then mice with a lower level of Ara9 protein expression would also display a phenocopy of the Ahr-null animal (i.e., a patent DV). We show here that these criteria can indeed be met. Furthermore, we provide the first evidence that Ara9 is essential for AHR-mediated developmental signaling.

Materials and Methods

Construction of the Ara9fxneo Targeting Vector. Creation of the Ara9fxneo targeting vector is identical to the creation of ΔCAra9fxneo and is described in Fig. 1 and in previous work from our laboratory (Lin et al., 2007). A BamHI restriction enzyme analysis resulting in fragment sizes of 553, 844, and 11,165 bp was performed to confirm the orientation of components of the targeting vector (PL 2008), including the tetratricopeptide repeat domains, Lox P sites, and arms of homology. Embryonic stem cell cultures and genotyping were performed using the same methods as reported previously (Lin et al., 2007). Visualization of the Ductus Venosus. Mice harboring a patent DV were identified by perfusion of the liver with a solution of trypan blue using methods described previously (Walisser et al., 2004a). The DV were identified by perfusion of the liver with a solution of trypan blue using methods described previously (Walisser et al., 2004a). The DV were identified by perfusion of the liver with a solution of trypan blue using methods described previously (Walisser et al., 2004a). The DV were identified by perfusion of the liver with a solution of trypan blue using methods described previously (Walisser et al., 2004a). The DV were identified by perfusion of the liver with a solution of trypan blue using methods described previously (Walisser et al., 2004a).

Western Blot Analysis. Western blots were performed using the methods described previously (Lin et al., 2007).

Statistics. In experimental analysis of multiple comparisons, an analysis of variance was performed, and Tukey’s test was used to determine differences, with a p ≤ 0.05. Statistical analysis of genotypic distribution was compared by χ² analysis (Devore and Peck, 1986).

Results

Generation and Genotyping of Ara9fxneo Mice. Homologous recombination of the Ara9fxneo targeting construct (Fig. 1A) into embryonic stem cells was confirmed by Southern blot analysis of BamHI-digested genomic DNA. The wild-type and Ara9fxneo recombinant allele gives rise to bands at 2.7 kb and 3.9 kb, respectively (Fig. 1B). Progeny resulting from a heterozygote by heterozygote cross were as follows: 12/52 (23%) were Ara9(+/+), 30/52 (58%) were Ara9(+/fxneo), and 10/52 (19%) were Ara9/ffxneo(fxneo) (Fig. 1C). The ratio of the genotypes obtained from χ² analysis was not significantly different from expected with a viable allele (p ≤ 1; χ² = 0.69) and indicated adherence to the expected Mendelian ratio of 1:2:1. Gross anatomy and histological examinations suggested that wild-type, heterozygote, and homozygous Ara9fxneo animals are outwardly normal (data not shown).

The Ara9fxneo Allele Is Hypomorphic. The expression of Ara9 was measured by Western blot analysis of protein extracts from heart, liver, kidney, spleen, and thymus from wild-type [Ara9(+/+)] and Ara9fxneo/ffxneo mice (Fig. 2A). Hypomorphs displayed marked decreases in Ara9 protein expression in all tissues examined. Antibodies raised against either the FK506-binding protein or tetratricopeptide repeat domains (Lin et al., 2007) yielded similar results (data not shown). Based on the Ara9 Western blot gradient, expression of the Ara9 protein from the Ara9fxneo allele was estimated to be approximately 10% of the expression of the wild-type allele (Fig. 2B).
The ARA9 Protein Is Necessary for Closure of the Ductus Venosus. To determine whether Ara9\textsuperscript{fxneo} mice display a patent DV similar to that observed in Ahr\textsuperscript{(--/--)} mice, we evaluated this phenotype by obtaining serial angiograms of contrast dye flowing through the portal vein and into the branches of the liver and then into the suprarenal then infrarenal IVC (Fig. 3A). In contrast, livers of Ahr\textsuperscript{(--/--)} and Ara9\textsuperscript{fxneo/fxneo} mice display portocaval shunting between the portal vein and IVC, indicating the presence of a patent DV (Lahvis et al., 2000). Perfusion assays using trypan blue were performed on a larger number of animals and revealed that Ara9\textsuperscript{fxneo/fxneo} mice display a patent DV at a frequency of 83%. Mice that are Ara9\textsuperscript{(+/-)} display a DV frequency of 56%. Mice that are Ara9\textsuperscript{(+/fxneo)} display a patent DV with a frequency of approximately 10% (Fig. 3B). Wild-type animals [Ara9\textsuperscript{(+/+)}) do not display a patent DV. No association was noted between the sex of the animal and persistence of the DV (data not shown). Estimates of DV patency in Ara9\textsuperscript{(--/--)} mice could not be obtained because these mice are resorbed or stillborn because of cardiac defects that begin around day E12 (Lin et al., 2007).

The Presence of DV Causes a Significant Decrease in Liver Weight. In addition to and as a result of the patent DV, another common phenotype observed in Ara9\textsuperscript{(--/--)} mice is a decreased relative liver weight. To investigate whether this relationship also holds true for the Ara9 hypomorphic lines, we compared relative liver weights from wild-type, heterozygous hypomorphic, homozygous hypomorphic, and heterozygous null animals. As expected, liver weight in Ara9\textsuperscript{fxneo/fxneo} and Ara9\textsuperscript{(+/-)} mice were significantly less than in wild-type and Ara9\textsuperscript{(+/fxneo)} mice (Fig. 3B).

Discussion

Characterization of the Ahr\textsuperscript{(--/--)} phenotype has defined a role for the AHR in a developmental pathway essential for the normal closure of the DV in the developing liver (Lahvis et al., 2000). Based on this observation, it follows that proteins that influence AHR folding, localization, or function may also be essential for AHR-mediated hepatovascular development. The simplest way to test this idea is to create mouse models with null alleles at candidate genes and to examine their AHR-mediated response profile. Unfortunately, most, if not all of the proteins known to be involved in AHR signaling are not amenable to classic gene targeting approaches that use null alleles, because many AHR-associated gene products are essential for more than one independent cellular pathway required for the survival of the animal. A relevant example is the ARNT protein, which is also required for developmental angiogenesis mediated by hypoxia-inducible factors (Maltepe et al., 1997; Chan et al., 1999). For ARNT, we were able to overcome its essential nature and provide evidence for its role in AHR-mediated development by using mice that exhibited decreased expression of ARNT (i.e., hypomorphic expression) (Walisser et al., 2004b). In
that model, we found that decreased expression of the ARNT protein allowed for normal embryonic angiogenesis, yet yielded a developmental phenotype that was identical to that observed in the Ahr(-/-) model (i.e., a patent DV).

Given our recent study demonstrating that Ara9(-/-) mice die in fetal development as a result of double-outlet right ventricle and ventricular septal defect, we chose to use a “hypomorph strategy” similar to that used for ARNT (Walisser et al., 2004b; Lin et al., 2007). To this end, we constructed a series of recombinant alleles at the Ara9 locus in mice. Our strategy was based on work from this laboratory and others, which has shown that targeted insertion of the Neo gene into a locus of interest frequently results in the generation of a hypomorphic allele (Walisser et al., 2004b). Given that Neo insertion was an intermediary step in generating a conditional Ara9 allele, a hypomorphic allele of Ara9 was constructed and used to characterize the influence of hypomorphic Ara9 expression on AHR-mediated liver development. It was our prediction the generation of a hypomorphic mouse would allow us to bypass embryonic lethality and enable us to study the effect of decreased Ara9 protein expression on AHR-mediated biology.

Using the Ara9fxneo allele along with our previously generated Ara9 allele, we created an allelic series where graded expression of the Ara9 protein could be achieved after appropriate genetic crosses. The expression levels of Ara9 protein ranged from highest to lowest in Ara9(+/-/+), Ara9(+/-/fxneo), Ara9(+/+), and Ara9(fxneo/fxneo) mice, respectively (Fig. 2A; data not shown). Although our Western blot-derived estimates of Ara9 protein expression from these models provides only a semiquantitative measure of relative protein expression, the data do support an estimate of approximately a 10% expression of Ara9 in the Ara9(fxneo/fxneo) mice, a 50% expression in the Ara9(+/-) mice, and a 60% expression in Ara9(+/-/fxneo) mice relative to wild-type mice (Fig. 2A).

Examination of a large cohort of Ara9 mutants supports our two initial predictions. First, we found that mice homozygous for a hypomorphic Ara9 allele (Ara9(fxneo/fxneo)) proceeded through development in normal numbers (Fig. 1), suggesting that 10% of normal expression of Ara9 is sufficient to overcome the block in heart development observed previously in null animals (Lin et al., 2007). Second, we hypothesized that a marked reduction in Ara9 expression would lead to insufficient AHR signaling and that the corresponding mice would display many of the phenotypes of Ahr-null animals. Examination of the developmental phenotype, patent DV, was of particular interest to us. To this point, we measured the frequency of the DV and found 83% of the hypomorphic animals to display this developmental phenotype (Fig. 3). In addition to the phenocopy exhibited by Ara9 hypomorphs and Ahr-null animals, additional support for the importance of Ara9 in AHR-mediated DV closure came from the observation of a “dose effect” of Ara9 expression on these endpoints. A slight reduction of Ara9 protein levels by one copy of Ara9fxneo (i.e., the Ara9(+/-/fxneo) model) led to only a 10% frequency of patent DV. Intermediate expression of Ara9 from the Ara9(+/-/) model display a patent DV incidence of 56%. Even greater reduction of Ara9 protein from two copies of the hypomorphic allele (i.e., Ara9(fxneo/fxneo) led to an 83% incidence of patent DV. In our examination of hundreds of wild-type mice (Ara9), we have never observed a patent DV. In keeping with this, we have observed a 0% frequency of patent DV in our wild-type cohort. Together, these data support the idea that Ara9 plays an important role in AHR-mediated developmental signaling.

Because of the persistence of the DV in Ara9 hypomorphic animals, this model has less power as a tool to study the adaptive and toxic pathways mediated by the AHR. This concept is based upon the observation that the portocaval shunting, which accompanies a patent DV, can lead to aberrant disposition of TCDD and related AHR agonists in many tissues, making a direct comparison between mutant and wild-type responses problematic (Thomae et al., 2004). For example, if we were trying to show a relationship between Ara9 expression and TCDD toxicity for a given liver endpoint, we would predict a decreased potency in the Ara9 hypomorphs. However, if this did occur, we would not know whether the attenuation was due to decreased cellular AHR signaling or to a decreased concentration of TCDD in the hepatic parenchyma as a result of shunting. Therefore, to understand the role Ara9 plays in toxic and adaptive signaling, our future plans include crossing the Ara9fxneo hypomorph with the FLPeR line (mice containing Flp-recombinase driven by the GTSRosa promoter), thereby allowing for germine excision of the neomycin cassette and creation of a conditional Ara9 mouse (Dymecki, 1996). These mice can then be crossed to mice where expression of Cre is driven by the Albumin promoter, allowing for specific excision of the Ara9 allele in hepatocytes (Kellendonk et al., 2000). Such a model will allow us to understand the relationship between hepatic expression of Ara9 and AHR-mediated hepatotoxicity and adaptive metabolism.

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References


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