Development and Characterization of a Transgenic Mouse Line Lacking the Dioxin-Binding Ah Receptor

Pedro Fernandez-Salguero, David M. Hilbert, Stuart Rudikoff, Jerrold M. Ward and Frank J. Gonzalez, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

1. Summary

The aryl-hydrocarbon receptor (AHR) has been the focus of considerable attention for many years since it mediates many carcinogenic and teratogenic effects of highly toxic environmental chemicals such as dioxins. An AHR deficient mouse line was constructed by homologous recombination in embryonic stem cells. A fraction of the mice homozygous for the receptor deficiency (Ahr-/-) die shortly after birth. The surviving Ahr-/- mice reach maturity and are fertile. A possible explanation for the decrease in survival is an observed significant delay and decrease in the accumulation of lymphocytes in the spleen and lymph nodes but not in the thymus of Ahr-/- animals. In addition, the livers of these mice are considerably smaller and have bile duct fibrosis and periportal hepatocyte eosinophilia. Ahr-/- mice are also nonresponsive to dioxin-mediated induction of genes encoding foreign compound-metabolizing enzymes. These results indicate that the AHR is important in liver development and in the acquisition of a normal immune system.

2. Introduction

The AHR constitutes the ligand-activated subunit of a unique heterodimeric transcription factor that is distinct from members of the steroid receptor superfamily [1]. It regulates cellular responses to a variety of highly toxic environmental chemicals such as dioxins (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD), benzo[a]pyrene in cigarette smoke and other combustion processes and polychlorinated and polybrominated biphenyls. A functional AHR appears to be required in laboratory animals for these environmental pollutants to cause cancer; mutagenesis; DNA adducts and oncogene activation; toxicity of the bone marrow, liver, eye and ovary; apoptosis of immature T cells; immunosuppression; atherosclerosis; endometriosis and birth defects [2-7]. Although many of these AHR-mediated effects probably occur in human populations, the epidemiology and other studies are mostly indirect [7,8].

The AHR belongs to the basic helix-loop-helix family of DNA binding proteins [1]. The basic region is responsible for sequence specific DNA binding, while the helix-loop-helix domain allows the AHR to interact with the Ah receptor nuclear translocator (Arnt) once bound to its ligand [9,12]. The heterodimer can thus interact with upstream regulatory sequences called AHR responsive elements (AHREs), xenobiotic responsive elements (XREs) or dioxin responsive elements (DREs) of an array of target genes, resulting in transcriptional activation [13,14]. Among the genes regulated through the AHR are those encoding the

cytochromes P450, CYP1A1, CYP1A2 and CYP1B1 [15-17] as well as phase II enzymes such as UDP-glucuronosyltransferase 1*06 (UGT1*06) [4].

The AHR is constitutively expressed in a large number of tissues in mammals, with highest levels found in liver, kidney, lung, heart and placenta [18-20]. Two or more AHREs have been identified in the regulatory domain 5' upstream of AHR regulated genes [21]. Variant alleles of the murine AHR have been found that differ in their binding affinity for polycyclic aromatic hydrocarbons [22,23].

An endogenous ligand has been postulated that could be involved with the AHR in development and homeostasis [24]. Involvement of the AHR in dioxin-mediated teratogenesis, apoptosis, immunosuppression and cell type-specific proliferation [5-7], indeed suggests that this transcription factor may play an important role in normal homeostasis. In the present study, the construction and characterization of a AHR-deficient mouse line is reported and the results strongly suggest that the AHR is involved in maintaining a normal immune system and is required for normal liver development.

3. Results and Discussion

Inactivation of the murine Ah receptor gene.

The murine AHR gene was inactivated in embryonic stem (ES) cells by homologous recombination [25] using a positive-negative selection strategy [26]. As shown in Fig.1, a 7.5 kb fragment from a 129/SV mouse genomic clone containing the coding exon 1 of the AHR was isolated, mapped and partially sequenced. Exon 1 encoding most of the basic region involved in DNA binding, was replaced with a neomycin resistance cassette containing the bacterial phosphoribosyltransferase II gene (neo) that is used as positive selection marker. The construct was ligated to a thymidine-kinase cassette (as negative selection marker) under the control of the herpes simplex virus promoter (HSV-TK).

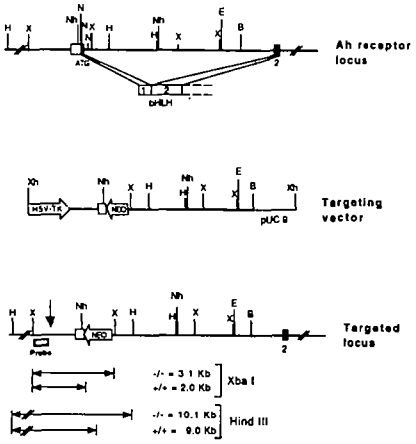

Figure 1.
Strategy for the inactivation of the murine AHR gene by homologous recombination. The vertical arrow indicates the 5' end of the 7.5 kb genomic fragment used in the construction of the targeting vector. The genomic probe used for screening is indicated by a shaded box. Coding exons are indicated by black boxes. The 5' untranslated region of the gene is indicated by an open box. bHLH stands for the basic helix loop helix domain of the AHR. Restriction endonucleases indicated: B, Bam HI; E, Eco RI; H, Hind III; N, Nar I; Nh, Nhe I; X, Xba I, Xh, Xho I.

The construct was linearized and electroporated into ES cells. Of 980 clones screened, three presented a legitimate homologous recombination event. These clones were injected into 3.5 days-old C57BL/6N mouse embryos. All the chimeras obtained showed a high 129/SV contribution as judged from coat color. The male chimeras were mated with C57BL/6N

females to give rise to heterozygote animals. These were interbred to produce an AHR homozygous mutant colony. Using tail genomic DNA, mice were screened for the presence of the targeted allele by Southern blot analysis.

Of 260 animals screened from heterozygous matings, we obtained a partially lethal phenotype. Between 40 to-50% of the homozygous mutant Ahr-/- mice died or were selectively cannibalized within 1 to 4 days after birth. Necropsy of these mice revealed lymphocyte infiltration of various organs, particularly the gut, urinary tract and lung. However, the precise cause of death could not be established. The frequency of genotypes obtained from Ahr+/- matings for wild type Ahr+/+, heterozygous Ahr+/- and homozygous mice were in accordance with a normal Mendelian distribution. Surviving Ahr-/- mice exhibited an apparent lower rate of growth within the first 2-4 weeks after birth as compared to Ahr+/+ or Ahr+/- littermates. Surviving mutant animals reached maturity and were fertile.

Expression of genes regulated by the Ah receptor in Ahr-/- mice.

TCDD-induced transcriptional activation of the CYP1A1, CYP1A2 and UGT1*06 genes is believed to be dependent on the Ah receptor [3,4,6,27-29]. CYP1A1 and CYP1A2, together with UGT1*06 are among the most relevant enzymes involved in the metabolism of foreign chemicals. CYP1A2 and UGT1*06 are constitutively expressed in liver while CYP1A1 is only expressed in this organ in the presence of inducer [30]. These genes are representative of at least six chromosomally non-linked mouse genes controlled by the AHR. We studied their constitutive and dioxin-inducible levels of expression (Fig. 2).

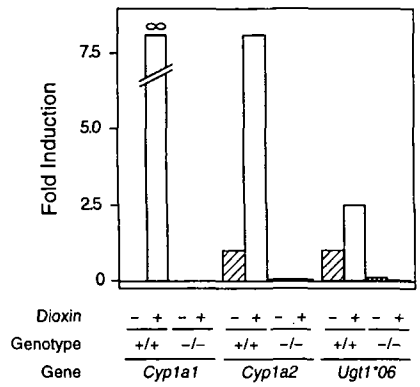

Figure 2.
Quantification by Northern (RNA) blot analysis of the expression of liver mRNAs encoding major AHR-regulated phase I and phase II enzymes. Six-week-old littermates were treated once with solvent (1,4-dioxane) or dioxin (TCDD) and total liver RNA was isolated and analyzed by Northern blots. The ∞ symbol stands for the maximum level of induction of CYP1A1 obtained in the liver of Ahr+/+ animals after dioxin treatment. This level of induction can not be related to the one in the absence of dioxin since no expression is detected without treatment.

Whereas Ahr+/+ animals exhibit a maximal level of induction of CYP1A1 in liver, the induction process in Ahr-/- mice is completely abolished. Similar results were obtained in lung and kidney. Liver CYP1A2 and UGT1*06 are also induced in Ahr+/+ animals after TCDD treatment by eight to ten fold and by two to three fold, respectively. No induction was observed in either liver or kidney in Ahr-/- mice. Furthermore, inactivation of the AHR not only abolished their inducible expression, but also dramatically down-regulates (by 85-90%) their liver-specific constitutive expression. These results suggest that the AHR, in the absence of exogenous ligand, controls basal gene expression and clearly indicates that either trans-

activation can occur on some genes in the absence of ligand or that an endogenous ligand occupies the binding site on the AHR allowing its interaction with certain AHREs.

Liver fibrosis in Ahr-/- mice.

The AHR is expressed in a large number of tissues in mice [19] and humans [20]. To understand the systemic effects of AHR deficiency, the major internal organs of Ahr-/- and Ahr+/+ mice were compared. Kidney, brain, heart, bone marrow, muscle, adrenal gland, thyroid and intestine showed no obvious histological abnormalities in Ahr-/- animals. In contrast, liver tissue from Ahr-/- mice consistently presents a unique phenotype that is both quantitatively and qualitatively different from liver tissues isolated from Ahr+/+ animals. Livers from 4-week-old Ahr-/- mice are only 2.9% ± 0.3% (n=15) of body mass as compared to 6.1% ± 0.4% (n=15) in Ahr+/+ or Ahr+/- animals. This difference in size is also consistantly observed in older (30 weeks) Ahr-/- animals. The general histological structure of the hepatic lobules are normal. However, Ahr-/- mice develop pronounced fibrosis in the portal tract, even though spontaneous liver fibrosis in mice is extremely rare and can be induced only by several hepatotoxins [31]. This phenomena is already observed in neonate Ahr-/- mice. They also display inflammatory changes in the bile ducts (cholangitis), eosinophilia in the periportal hepatocytes that resembles that seen after treatment of mice with xenobiotics, centrilobular hypercellularity, glycogen depletion and ductular hyperplasia. Additionally, as Ahr-/- mice age (25-30 weeks) perivascular fibrosis, osseous metaplasia, pneumonia and plasma cell cuffs are also observed in their lungs. Several older (30-35 weeks) Ahr-/- mice showed severe bronchopneumonia and lung congestion. Nevertheless, these pathologies were not yet lethal in the oldest animals studies and in the absence of treatment with dioxin. These results suggest that the histological pathologies, in addition to the reduction in the expression of enzymes that are essential for xenobiotic detoxification, might impair the liver function in Ahr-/- mice, possibly contributing to their lower rate of growth at early ages (2-4 weeks). The possibility that the AHR protects the organism from endogenous or even dietary chemicals during development should also be considered.

Immune system impairment in Ahr-/- mice.

The role of the AHR in benzo[a]pyrene-induced suppression of B cell lymphopoiesis [32], in dioxin-induced alterations of the host response to infections [33] and in other forms of immunosuppression [34] have been postulated. However, to date, no evidence has been reported to directly link the AHR to such a putative network. To evaluate this hypothesis, we assessed the cellular composition and the histology of thymus and spleen in Ahr-/- and Ahr+/+ mice. The thymus is histologically undistinguishable between wild type and homozygous mutant animals. In contrast, the spleen of 4-week-old Ahr-/- mice displays significantly smaller, although structurally normal, periarterial lymphatic sheaths (PALS) as compared to age-matched Ahr+/+ animals. A quantitative comparison of splenic lymphocyte numbers in Ahr+/+ and Ahr-/- mice indicates that this difference varies with the age of the animals (Fig. 3, Panel A). In particular, young Ahr-/- mice (2-3 weeks) contain 10-15% the number of lymphocytes than do Ahr+/+ littermates. As Ahr-/- mice get older, the number of splenic lymphocytes increases gradually reaching normal levels by 10-12 weeks after birth. Among the oldest mice tested (25-30 weeks) there is a significant decrease in splenic lymphocyte numbers such that Ahr-/- mice have 20-30% the number of cells than do Ahr+/+ controls. Similar analyses of peripheral lymph nodes (LN) from normal and mutant mice (data not shown) indicates that Ahr-/- LN also contain significantly fewer lymphocytes than Ahr+/+ nodes, suggesting that AHR deficiency results in a developmental delay in the seeding of peripheral lymphoid tissues. This delay appears restricted to the peripheral immune

system since no discernible differences were observed in either the absolute number (Fig. 3, panel A) or the cell surface phenotype (data not shown) of cells taken from a generative organ of the immune system, the thymus.

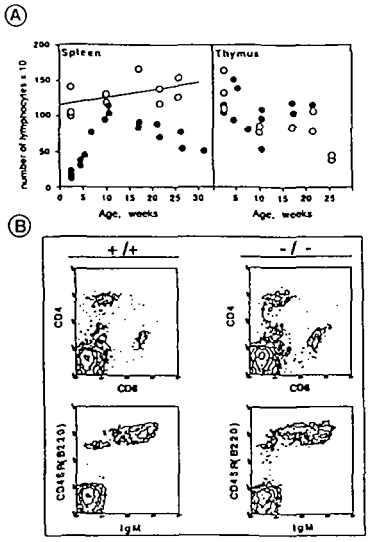

Figure 3.
Flow cytometry analyses of wild type and homozygous mutant mice. (A) Number of lymphocytes recovered from the spleen and thymus of Ahr+/+ (0) and Ahr-/- (●) mice. (B) Distribution of T and B cell populations in the spleen of Ahr+/+ (+/+) and Ahr-/- (-/-) mice. CD4 and CD8 quantify T cells whereas CD45R(B220) and IgM quantify B cells. The same animals where used in both parts of panel A. Flow cytometry measurements were performed on single cell suspensions of spleen, lymph nodes and thymus obtained from Ahr+/+ and Ahr-/- littermates. All monoclonal antibodies were directly conjugated to either phycoerythrin (PE) or Flourescein-isothiocyanate (FITC). Measurements were obtained with a Becton-Dickinson FACSCAN.

In view of the clear discrepancy in the absolute number of peripheral lymphocytes in young Ahr+/+ and Ahr-/- mice, it was of interest to determine if this difference was due to the absence of a specific lymphocyte subpopulation, or to a systemic AHR mediated defect in the ability of lymphocytes to reside in the periphery. Accordingly, lymphocytes from spleen and LN from normal and mutant mice were analyzed for the expression of lymphocyte specific, cell surface markers (Fig 3, Panel B). The staining pattern for T and B cells between spleens obtained from representative 2-week-old Ahr+/+ and Ahr-/- mice are virtually identical. In all mice tested, the expected proportion of T cells, as assessed by the expression of CD4 (3-6%) and CD8 (2.5%) were obtained. Expression of the B cell specific markers CD45R(B220) (19-38%) and IgM (15-30%) are also comparable in Ahr+/+ and Ahr-/- mice. Similar analyses of LN cells (not shown) also revealed no significant differences in the proportion of T and B cells. Immunohistochemistry studies performed on frozen sections of spleen and thymus also indicate no significant difference in the proportion of T and B cells in either organ between Ahr-/- and Ahr+/+ mice (not shown). These results suggest that AHR deficiency leads to a general delay in the appearance of peripheral T and B lymphocytes.

The mechanisms by which AHR deficiency delays the appearance of peripheral lymphocytes, and subsequently decreases their number among mutant animals remains unclear. However, it is plausible that AHR deficiency may alter the normal process of positive selection within the thymus and bone marrow thereby limiting the number of lymphocytes emigrating to the periphery. Such a scenario would allow for the generation of apparently normal numbers of progenitor B and T cells but only a small fraction would successfully emigrate to the periphery. Alternatively, AHR deficiency may not alter the production of competent lymphocytes but rather interfere with their ability to efficiently home to the appropriate peripheral lymphoid organ. Those cells displaying inappropriate homing

specificities would be subject to elimination. Finally, AHR deficiency may simply shorten the normal lifespan of peripheral lymphocytes. In young animals who generate large numbers of immune cells, a shortened half-life may only delay the accumulation of peripheral lymphocytes. In contrast, older mice whose production of newly emerging lymphocytes has diminished, would likely experience a decrease in the absolute number of peripheral lymphocytes as a result of a shortened lymphocyte lifespan. Since the liver is the organ involved in hematopoiesis during fetal development and Ahr-/- mice already present liver fibrosis and low number of lymphocytes at birth, we investigated the hepatic content of T and B lymphocytes during development. Preliminary results indicate that the liver of Ahr-/- mice produce fewer lymphocytes than Ahr-/+ control animals at the same gestational stage. To clarify the possible lack of functionality of these cells, lymphocyte transfer from Ahr-/- fetal liver into immunocompromised mice is currently underway. Similarly the premature decrease in immunocompetence in older Ahr-/- mice is being assessed by the transfer of T and B cells precursors from the bone marrow of Ahr-/- mice to Rag-2 immunocompromised animals. In either case, the viability of these lymphocytes will be clarified.

These results suggest that the absence of the AHR in mice could compromise their ability to fight infections and external insults that, in certain situations, might result in a threat to survival. We believe that this mouse line will be invaluable for carcinogenesis and chemical risk assessment studies involving such highly toxic environmental pollutants as dioxin, benzo[a]pyrene in cigarette smoke and other combustion processes, polychlorinated and polybrominated biphenyls, arylamines and other occupational hazardous agents, and drugs. The effects of these toxic chemicals in the liver, lung and in the immune system without the mediation of the AHR will help clarify the genotoxic and non-genotoxic pathways for tumor initiation, tumor promotion and progression, mutagenesis and toxicity. In addition, this mouse line will help delineate the role of the AHR in normal liver and immune system development.

4. References

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