TUMOR SUPPRESSOR FUNCTION OF ARYL HYDROCARBON RECEPTOR (AHR): EARLY ONSETS OF SPONTANEOUS LYMPHOMA AND HEPATOMA, AND RESULTING SHORTENED LIFE SPAN OBSERVED IN AHR DEFICIENT MICE

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Introduction

Studying the biological function of aryl hydrocarbon receptor (AhR) elucidates a unique view of the mechanism underlying physiological modification of endocrine systems by AhR-ligands other than dioxin-related chemicals, for example, the recent discovery of bi-lateral, anti-estrogenic and estrogenic, estrogen-receptor functions as an AhR cofactor depending with or without estradiol[1, 2]. AhR induces epigenetic carcinogenesis via the activation of cytochrome P450 1A1 (Cyp1A1) drug metabolism pathway. Consequently, constitutive active AhR transgenic mice showed high incidence of liver tumor induced by N-nitrosodiethylamine[3]. However, contrarily to the above-mentioned promoting function of AhR, we have found that it has a tumor suppressor function. AhR-mediated xenobiotic signals induce G_1 cell cycle arrest[4, 5], although the question of whether the induction of the $p27^{KIP1}$/cdk inhibitor by such signals is direct or indirect, remains unanswered[6, 7]. AhR knockout (KO) mice nullify the benzene-induced hematotoxicities[8], and show cell cycle acceleration in primitive hemopoietic progenitor cell compartments[9]. The onset of spontaneous neoplasms and resulting shortened life span were preliminarily determined from our laboratory meta-data. Thus, AhR may function as a plausible suppressor of tumorigenesis. The present results show Cyp1A1- independent, AhR-mediated tumor suppressive function of the AhR for the first time.

Materials and Methods

Animals. The generation of the homozygous AhR KO ($AhR^{-/-}$) mice, the 129/SvJ strain, is described elsewhere[8, 10]. $AhR^{-/-}$ mice were maintained in a board-approved laboratory animal facility of the National Institutes of Health Sciences (NIHS) of Japan. Heterozygous AhR KO ($AhR^{+/-}$) males were backcrossed with C57BL/6 females. Breeding 12 generations of heterozygous AhR KO ($AhR^{+/-}$) males with $AhR^{+/-}$ females generated wild-type ($AhR^{+/+}$), $AhR^{+/-}$, and $AhR^{-/-}$mice, although incidence of $AhR^{-/-}$mice resulted in a bit lower than Mendelian estimate. The neonates were genotyped by a polymerase chain reaction (PCR) screening of DNA from the tail. All the mice were housed under specific pathogen-free conditions at 24 ± 1°C and 55 ± 10% R.H., using a 12-hr light-dark cycle. Autoclaved tap water and food pellets were provided *ad libitum*.

PCR for genotyping. To detect AhR wild-type and AhR-KO alleles, PCR was performed using genomic DNA extracted from the tail of each mouse or from tumors cells of the mice in the carcinogenesis tests, and synthetic oligonucleotides were used as primers[10]. To detect the AhR wild-type allele, the 5' common primer (cgcgggcaccatgagcagc) and the 3' primer (cgcattggttgagactcag) were used; to detect the AhR-KO allele, the 5' common primer and *LacZ*-primer (cgccgagttaacgccatcaa) were used.

Life-time observation. Twenty-four mice of $AhR^{+/+}$, 23 of $AhR^{+/-}$, and 19 of $AhR^{-/-}$male mice were used in this study. All the mice were monitored throughout their lifetime at least twice daily. Those showing symptoms of advanced leukemia such as anemia and palpable splenomegaly were euthanized at the agonal period and then examined hematopathologically. Additionally, mice that died were subjected to gross and microscopic examination.

Histopathological examination. To evaluate hemopoietic malignancies, mice from each group sacrificed under ethyl ether anesthesia and autopsied. For histopathological examination, all the visceral organs of the mice including the thymus, spleen, sternum, and femoral bone marrow (BM) were fixed in 10% neutral buffered formalin for 24h. The sternum and femoral BM were decalcified in 7.5% formic acid for 72h. After conventional processing for dehydration, paraffin-embedded sections were stained with hematoxylin and eosin (H and E) and then examined histopathologically under a light microscope[11, 12].

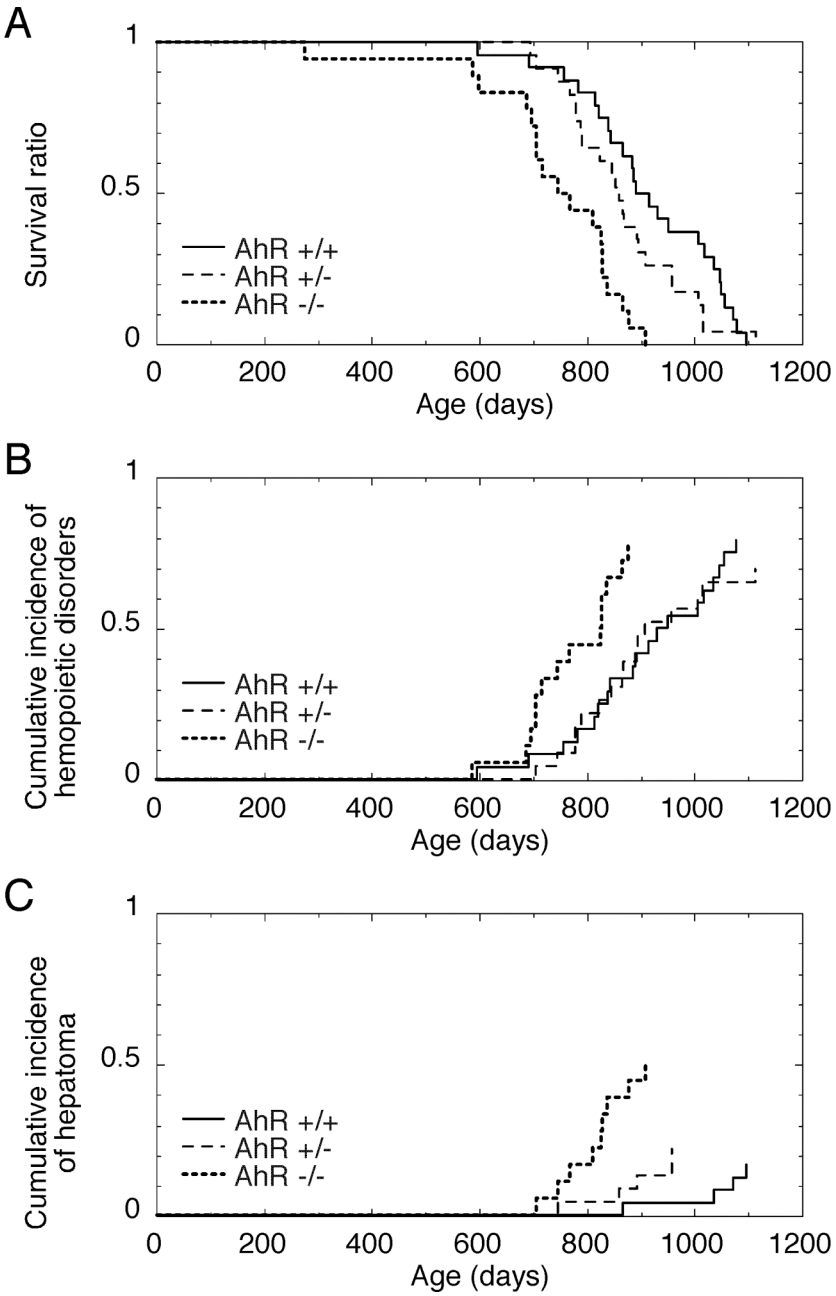

Figure 1. Survival curves (A), cumulative incidence of hemopoietic disorders (B) and cumulative incidence of hepatoma (C) for the $AhR^{-/-}$, $AhR^{+/-}$ and wild-type groups.

Table 1: Histopathological findings of the $AhR^{-/-}$, $AhR^{+/-}$ and wild-type groups.

Genotype	AhR +/+		AhR +/-		AhR -/-	
Number of mice	24	(100.0%)	23	(100.0%)	18	(100.0%)
Hemopoietic disorders	19	(79.2%)	16	(69.6%)	14	(77.8%)
Non-thymic lymphoma	19	(79.2%)	14	(60.9%)	10	(55.6%)
Malignant fibrous histiosarcoma	0	(0.0%)	1	(4.3%)	3	(16.7%)
Fibrosis of the BM	0	(0.0%)	1	(4.3%)	1	(5.6%)
Hepatoma	4	(16.7%)	5	(21.7%)	9	(50.0%)
Other tumor*1	3	(12.5%)	3	(13.0%)	2	(11.1%)
Tumor free death*2	2	(8.3%)	4	(17.4%)	1	(5.6%)
Number of tumors/mouse*3	1.18	–	1.26	–	1.47	–

*1: Other tumors include lung cancer, hemangiosarcoma of the spleen, adenoma of the stomach and rectal cancer.
*2: Tumor free deaths include nephrosclerosis, sepsis, and polycystic kidney.
*3: Number of tumors/mouse (multiplicity) is calculated as a function of total number of tumors observed per mice, which carried at least one tumor.

Results and Discussion

C57BL/6 $AhR^{-/-}$, $AhR^{+/-}$, and wild-type mice from the same litter were used in this study. Groups of nineteen $AhR^{-/-}$, 23 $AhR^{+/-}$, and 24 wild-type male mice were observed throughout their lifetimes and histopathologically examined after death or euthanized sacrifice at the agonal phase if mice showed symptoms of advanced leukemia. **Figure 1A** (at the top) shows the survival curves for the $AhR^{-/-}$, $AhR^{+/-}$ and wild-type groups from left to right, respectively. The mean life span of the $AhR^{-/-}$ group was 756 days, which was significantly shorter than the mean life span of both $AhR^{+/-}$ (854.5 days) and wild type (890 days) groups .The difference was statistically significant by the Kaplan Meier/Log Rank test ($P<0.002$ and $p<0.00001$, respectively). Histopathological examination confirmed the mechanism underlying the shortened life span observed in the $AhR^{-/-}$ group. **Figure 1B,** in the middle, shows the early onset of hemopoietic neoplasms in the $AhR^{-/-}$ group, which started before the 600th day. The histopathological subtypes of hemopoietic neoplasms are shown in the **Table 1**. In the cases of non-thymic lymphoma, the total incidence in the $AhR^{-/-}$ group was not significantly high compared with that in the $AhR^{+/-}$ or wild-type group, thus, early on set of non-thymic lymphoma in the $AhR^{-/-}$ group compared with those in the $AhR^{+/-}$, and wild-type groups may be due to higher impact of neoplastic promotion in the $AhR^{-/-}$ mice. In the case of hepatoma shown in **Figure 1C** (at the bottom), both the onset and total incidence respectively were early and high in the $AhR^{-/-}$ group compared with those in the $AhR^{+/-}$, and wild-type groups ($P<0.00002$, $p<0.000002$ for onset, Kaplan Meier/Log Rank test: $P=0.0592$, $p=0.0482$ for incidence, Fisher's exact probability test, respectively). Careful histopathological examination provided essentially no significant histological differences among the hepatomas in each AhR genotype except a slightly lesser extent of differentiation in $AhR^{-/-}$ hepatoms than that in $AhR^{+/+}$ wild type hepatomas.. The mechanism underlying the split of neoplastic behavior between hemopoietic neoplasms and hepatomas suggests their different cause of neoplastic development with respect to the AhR function. As a result, we observed a slight decrease in tumor-free death in the $AhR^{-/-}$ group (5.6% compared with 17.4% in the $AhR^{+/-}$ and 8.3% in the wild-type groups). Furthermore, multiplicity of tumors per mice is increased along with the AhR gene dosage manner. Onsets of hepatoma in the $AhR^{+/-}$ and wild-type mice were delayed compared to that of the $AhR^{-/-}$ mice, which made lower incidence in hepatoma in both groups, probably due to competitive risk from the hematopoietic neoplasms.

In summary, the $AhR^{-/-}$ mice exhibited an early onset of spontaneous neoplasms, that is, lymphoma and hepatoma, and consequently had a shorter life span than the $AhR^{+/-}$ and wild-type mice. Because of the early onsets of lymphoma and hepatoma observed in the $AhR^{-/-}$mice, implying that the biological function of AhR are supposed to be tumor suppression based on physiological cell cycle stabilization.

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