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AhR SUPPRESSES HEMOPOIESIS DURING STEADY STATE BUT ACCELERATES CELL CYCLE AS AN EARLY RESPONSE: A STUDY OF AhR-KNOCKOUT MICE.

Yoko Hirabayashi[1], Guang-Xun Li[1], Byung-Il Yoon [1],
Yoshiaki Fujii-Kuriyama[2], Toyozo Kaneko[1], Jun Kanno[1] and Tohru Inoue[3]

[1]Division of Cellular & Molecular Toxicology, and [3]Center for Biological Safety and Research, National Institute of Health Sciences, Tokyo 158-8501, Japan.
[2]Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Tsukuba 305-8577, Japan.

Introduction

Whether the aryl hydrocarbon receptor (AhR) physiologically suppresses cell growth through relevant signals via a possible endogenous ligand[1] or facilitates cell cycle progression from G_1 with *Fos* and *Jun* signaling, is still controversial[2]. In this study, the authors attempted to elucidate a possible consistent function of AhR in hematopoiesis using AhR-knockout (KO) mice[3].

As the authors focused on B cell suppression during B lymphopoiesis[4], the effects of TCDD exposure on hemopoiesis were extensively investigated, since the inhibitory effects of TCDD on bone marrow and immunological parameters, including granulocyte-macrophage (GM) colony forming unit (CFU) and other progenitors, were first recognized by Luster and coworkers in the early 1980's[5, 6]. The down-regulation of AhR expression attenuates myelosupression in thioredoxin (Trx/ADF)-overexpressing mice, as determined by hemopoietic colonization assay, which elucidated the linkage of AhR signals to the antioxidant cascade induced by reactive oxygen species, ROS, after TCDD exposure[7, 8].

In this study, attention was focused on the function of AhR in the hemopoietic system, specifically in hemopoietic stem cells/progenitor cells, and the controversial dual function of AhR was found to be consistent because AhR seems to stimulate the cell cycle as an early response to cytokines, whereas simultaneously, suppress hematopoiesis during the steady state. The dual function of AhR found in the present study may contribute to a better understanding of health effects that can be induced by an interaction between AhR and its environmental ligands.

Materials and Methods

Animals. The establishment of homozygous AhR KO ($AhR^{-/-}$) mice, 129/SvJ strain, is described elsewhere[3, 9]. The breeding of heterozygous AhR KO ($AhR^{+/-}$) males with $AhR^{+/-}$ females generated wild-type ($AhR^{+/+}$), $AhR^{+/-}$, and $AhR^{-/-}$mice. The neonates were genotyped by PCR screening of DNA from the tail. Female mice (12 weeks old) were used in the study. Eight-week-old C57BL/6 male mice from Japan SLC (Shizuoka, Japan) were used as recipients for the CFU assay in the spleen. All the mice were housed under specific pathogen-free conditions at 24 ± 1 °C and 55 ± 10%, using a 12-hr light-dark cycle. Autoclaved tap water and food pellets were provided *ad libitum*.

Blood and bone marrow (BM) parameters. Peripheral blood was collected from the orbital sinus. Peripheral blood leukocyte (WBC), red blood cell (RBC) and platelet numbers were measured using a blood cell counter (Sysmex M-2000, Sysmex Co., Kobe, Japan). BM cellularity was evaluated by harvesting BM cells from the femurs of each mouse[10]. The animals were sacrificed. Then a 27-gauge needle was inserted into the femoral bone cavity through the proximal and distal edges of the bone shafts, and the BM cells were flushed out under pressure by injecting

2 ml of DMEM without phenol red. A single-cell suspension was obtained by gently and repeatedly triturating the BM cells through the 27-gauge needle and the cells were then counted using Sysmex M-2000.

Irradiation. Recipient mice were exposed to a lethal radiation of 915 cGy, at a dose rate of 124 cGy/min using a ^{137}Cs-gamma irradiator (Gamma Cell 40, CSR, Toronto, Canada) with a 0.5-mm aluminum-copper filter.

CFU-S Assay. The Till and McCulloch method[11] was used to determine the number of colony-forming unit in the spleen (CFU-S). Aliquots of BM cell suspensions were used to evaluate the number of CFU-S. The number of BM cells was adjusted to that appropriate for producing nonconfluent spleen colonies, and the cells were then transplanted into lethally irradiated mice by injection through the tail vein. Spleens were harvested 9 and 13 days later, and fixed in Bouin's solution. Macroscopic spleen colonies were counted under an inversion microscope at a magnification of ×5.6.

CFU-GM and CFU-E Assay. *in vitro* colony formation was assayed in semisolid methylcellulose culture. Briefly, 8×10^4 BM cells suspended in 100 µl of medium were added to 3.9 ml of a culture medium containing 0.8% methyl-cellulose, 30% fetal calf serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, with 10 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF) for CFU-GM or 1ng/ml murine Interleukin-3 and 2 U/ml erythropoietin for erythroid CFU (CFU-E). One-ml aliquots containing 2×10^4 BM cells were plated in triplicate in a 35-mm tissue-culture plate, and incubated for six days in a completely humidified incubator at 37 °C with 5% CO_2 in air. Colonies were counted under an inverted microscope at a magnification of ×40 for CFU-GM after 6-day culture and ×100 for CFU-E after 3-day culture.

BUUV assay. The bromodeoxyuridine (BrdUrd)-labeled cells purged by ultraviolet light to evaluate the kinetics of hemopoietic progenitor cells, BUUV assay was employed, as described elsewhere[10, 12]. Briefly, cells in the S phase (DNA synthesis) were labeled *in vivo* with BrdUrd followed by exposure to near-ultraviolet light in order to kill cells that incorporated BrdUrd, and then survival ratio was evaluated based on the numbers of hemopoietic colonies (CFU-GM and CFU-S) that form during the assay.

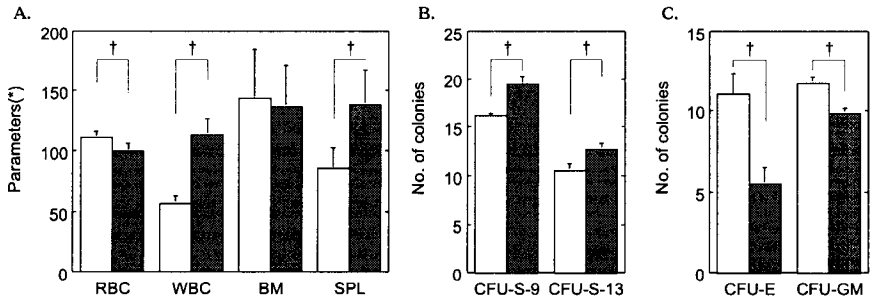

Figure 1: Comparison of various blood parameters between wild-type mice (open bars) and $AhR^{-/-}$ mice (shaded bars); **A.** Peripheral blood, bone marrow and spleen weight. * Parameters indicate the counts of peripheral red blood cells (RBCs, $\times10^8$/ml) and white blood cells (WBCs, $\times10^6$/ml), bone marrow cellularity (BM, $\times10^5$/femur), and weight of the spleen (SPL, mg). **B.** Number of colony-forming unit in spleen (CFU-S/1×10^5 BM cells) observed on days 9 (CFU-S-9) and 13 (CFU-S-13). **C.** Numbers of *in vitro* granulocyte-macrophage CFU (CFU-GM/5×10^3 BM cells) and erythroid CFU (CFU-E/1×10^4 BM cells). †: Significantly different by *t*-test between $AhR^{+/+}$ and $AhR^{-/-}$ mice at $p<0.05$.

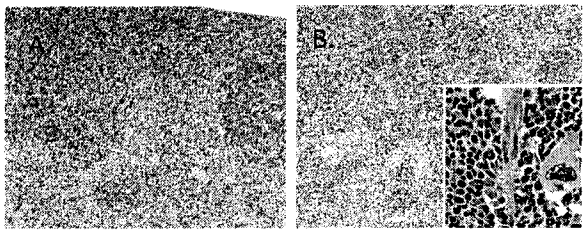

Figure 2: Histological findings on the spleen from $AhR^{+/+}$ mice (A) and $AhR^{-/-}$ mice (B). Note, a prominent enhancement of hemopoiesis in the spleen can be observed in $AhR^{-/-}$ mice (B). (HE staining. Magnification: A&B ×20, the inset of B ×80.)

Results and Discussion

$AhR^{-/-}$ mouse shows significant increase of hemopoiesis: WBC increases in the $AhR^{-/-}$ mice (Fig. 1A). This is the first observation found in the $AhR^{-/-}$ mice in the present study that is consistent with the hypothetical description by Adachi and coworkers[1] in which a possible physiological ligand is, speculated to suppress hemopoiesis in the $AhR^{+/+}$ mice. This is also consistent with the higher number of myeloid progenitor cells, *i.e.*, CFU-S-9 and CFU-S-13, observed in the $AhR^{-/-}$ mice (Fig. 1B). Thus, steady-state hemopoiesis is presumed to be suppressed via AhR signaling by a possible physiological ligand, which is not readily observed in $AhR^{-/-}$ mice. In response to such AhR-null effect, the $AhR^{-/-}$ mouse reversely shows extensive hemopoiesis in the spleen (Fig. 2B), although this hemopoietic enhancement is also reflected in another negative hemopoietic regulation in the BM.

AhR promotes cell cycling in hemopoietic progenitors: Interestingly, when BM cells are removed from $AhR^{-/-}$ mice as well as from $AhR^{+/+}$ mice, and are grown in *in vitro* colony assay, the numbers of CFU-GM and CFU-E are both significantly lower in the $AhR^{-/-}$ mice (Fig. 1C), implying that AhR signaling promotes of acute phase response for cytokines during colony growth. The decrease in the number of CFU-E in Fig. 1C possibly affects the number of RBCs in the peripheral blood of the $AhR^{-/-}$ mice (Fig. 1A). These observations were consistent with those in the case of other hemopoietic progenitors (data not shown).

Cell kinetics of CFU-GM receives negative feedback in steady state: The BUUV method* shows a clear enhancement of the cell cycle in primitive progenitor cells, CFU-S-13 (Fig. 3A) and relatively mature progenitor cells, CFU-S-9 (Fig. 3B) in the $AhR^{-/-}$ mouse. Although the precise mechanism underlying this phenomenon is not known yet, the cell kinetics of CFU-GM becomes rather suppressed in terms of percent cycling fraction per unit time, *i.e.*, less than 5% (Fig. 3C), which may be due to a possible negative feedback to an up-regulated cell kinetics of primitive progenitors (Figs. 3A and 3B). Since the number of WBCs in the peripheral blood was higher in the $AhR^{-/-}$ mice, the present data on CFU-GM in Fig. 3C seem to be incomplete, and presumably, the number of steady-state CFU-GM for the $AhR^{-/-}$ mouse (closed circles) may be higher than that for the wild-type mice.

The lack of AhR and the complex compensation of BM hemopoiesis might still be insufficient for the $AhR^{+/+}$ mice, because a compensatory increase in splenic weight in the $AhR^{-/-}$ mice is evident (Figs. 1A and 2).

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* Continuous perfusion of BrdUrd through an osmotic pump permits to obtain a variety of parameters in the cell kinetics of the hemopoietic progenitor cell compartment, such as a doubling time, a size of cycling- or quiescent fraction, and also the size of cycling fraction during the unit time-interval.

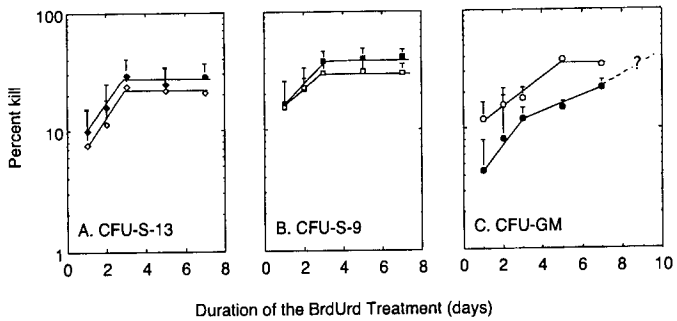

Figure 3: Hemopoietic progenitor cell kinetics of each hemopoietic progenitor compartment from $AhR^{+/+}$ mice (open symbols) and $AhR^{-/-}$ mice (closed symbols), measured by BUUV method (see Materials and Methods); Number of colony forming units in spleen observed on days 13 (CFU-S-13, **A**.) and 9 (CFU-S-9, **B**.), and number of granulocyte-macrophage CFU (CFU-GM, **C**.). CFU-S-13 and CFU-GM are significantly different by a paired *t*-test between $AhR^{+/+}$ and $AhR^{-/-}$ mice ($p<0.05$). The plateau level (between day 3 and day 7 of BrdUrd treatment) of CFU-S-9 is also significantly different by a *t*-test between $AhR^{+/+}$ and $AhR^{-/-}$ mice ($p<0.05$)).

In conclusion, as reported by Puga and coworkers[2], AhR functions as a cell-cycle regulator rather than a drug-metabolizing enzyme inducer; thus, possible phenotypes transmitted via AhR may be diversifiable.

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