AHR-MEDIATED BENZENE-INDUCED HEMATOPOIETIC TOXICITIES: DIFFERENTIAL TOXICITIES BETWEEN ONE FROM AHR IN THE HEMATOPOIETIC STEM CELLS FOR THE BONE MARROW AND THE OTHER FROM POSSIBLE HEPATIC-AHR FOR THE PERIPHERAL BLOOD

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Introduction

The elucidation of the signal transduction mechanism associated with the aryl hydrocarbon receptor (AhR) after benzene exposure may contribute to understanding the physiological role of the AhR during xenobiotic responses mediated by the AhR. Recently, we have found that the benzene-induced hematopoietic toxicity was transducerd via the AhR, and the toxicity was attenuated when the AhR-knockout (KO) mouse was exposed to benzene[1]. Consequently, a question is raised on whether the benzene-mediated hematotoxicity is derived from the primitive hematopoietic progenitor cells in the bone marrow (BM) or from classic hepatic xenobiotic metabolism, because the AhR expression was found to be high in the primitive hematopoietic progenitor cells[2,3], which were reported to express CYP450-2E1[4]. To answer the question, BM transplantation assays were; first, BM cells from wild-type mice or AhR-KO mice were utilized to repopulate lethally irradiated wild-type mice, and second, the wild-type BM cells were transplanted into lethally irradiated wild-type or AhR-KO mice, then, the possible changes in the steady state among the groups were compared one month after repopulation followed by benzene exposure. The first part of the experiments has been preliminarily reported[5], and presently, the succeeding experiments have been finished; thus, the entire results are discussed in this paper.

Materials and Methods

Experimental animals. The generation of homozygous AhR-KO ($AhR^{-/-}$) mice of the 129/SvJ strain has been described elsewhere [1, 6]. The animal experiment using $AhR^{-/-}$ mice was approved by the Interdisciplinary Monitoring Committee for the Proper Use and Welfare of Experimental Animals. The 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. The breeding of over 20 generations of heterozygous $AhR^{+/-}$ males with $AhR^{+/-}$ females generated wild-type ($AhR^{+/+}$), $AhR^{+/-}$, and $AhR^{-/-}$mice, although the proportion of $AhR^{-/-}$ mice was slightly lower than the Mendelian estimate. The neonates were genotyped by the 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%, room temperature and humidity, respectively, using a 12-hr light-dark cycle. Autoclaved tap water and food pellets were provided *ad libitum*.

Benzene and benzene exposure. Benzene, CAS. No. 71-43-2, MW 78.11, was purchased from Wako Fine Chemical Company (Osaka, Japan). Experimental mice were intragastrically (*i.g.*) administered with freshly prepared corn oil solutions of benzene (150 mg/kg body weight) once daily for 5 days/week for 2 weeks. The dose used in this study was 150 mg/kg body weight, which corresponds to the daily dose for leukemia induction, i.e., 300 ppm for 6 hr/day, 5 days/week for 26 weeks[7, 8].

Blood and bone marrow (BM) parameters. Peripheral blood was collected from the orbital sinus. Peripheral blood leukocyte (WBC) and red blood cell (RBC) counts were determined using a blood cell counter (Sysmex K-4500, Sysmex Co., Kobe, Japan). BM cellularity was evaluated by harvesting BM cells from the femurs of each mouse[9]. 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 BM cells were flushed out under pressure by injecting 2 ml of α-MEM. A single-cell suspension was obtained by gently triturating the BM cells through the 27-gauge needle, and the cells were counted using Sysmex K-4500.

CFU-GM Assay. *In vitro* colony formation was carried out in semisolid methylcellulose culture[9, 10] Briefly,

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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). One milliliter 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 magnifications of ×40 after 6-day culture.

Irradiation. In the BM repopulation assay, recipient mice were exposed to a lethal radiation of 800.1 cGy, at a dose rate of 103.5 cGy/min, using a ^{137}Cs-gamma irradiator (Gamma Cell 40, CSR, Toronto, Canada) with a 0.5-mm aluminum-copper filter.

***BM repopulation assay*[11].** In the BM repopulation assay, a single-cell suspension of BM (1 x 10^6 cells) from AhR-KO or wild-type litter mice was injected into the tail vein of 800.1 cGy-irradiated, AhR-KO or wild-type litter recipient mice. One month after the transfusion of BM cells, the repopulated mice were used in the experiment.

Results and Discussion

Steady-state hematopoiesis before and after BM exchanges between wild-type and AhR-KO mice after lethal-dose whole-body irradiation. **Figure 1A** shows comparisons of steady-state numbers of RBCs, WBCs, and BM cells, and the splenic weight between wild-type mice (solid columns) and AhR-KO mice (open columns) (without BM exchange or benzene treatment). AhR-KO mice show a more significant decrease in the number of RBCs, and a more significant increase in number of WBCs. They also show a more significant increase in the weight of spleens. The reason for the significant decrease in the number of RBCs in AhR-KO mice is unknown. The significant increase in the splenic weight and number of WBCs in AhR-KO mice are attributable to the increased hematopoiesis in the spleen, which is considered to be caused by the increased number of primitive hematopoietic progenitor cells (data not shown[2]) and consequent decrease in the number of CFU-GM in the BM[2]. From the results, we conclude that the AhR, which functions as a repressor of cell cycling in the primitive hematopoiesis, may contribute to restoring the stem cell compartments quiescent[12].

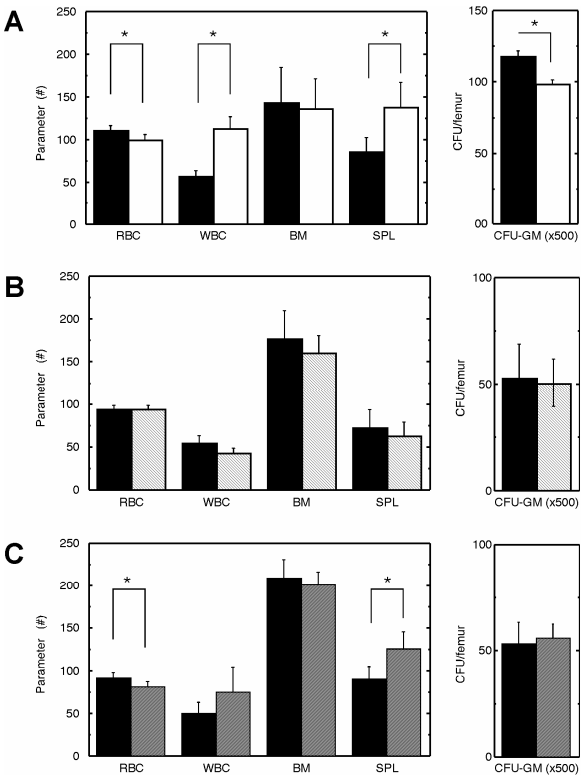

Figure 1: Comparison of various blood parameters between **A**, wild-type mice (solid columns) and AhR-KO mice (open columns), **B**, wild-type mice repopulated with wild-type BM cells (solid columns) or AhR-KO BM cells (light-shaded columns) **C**, wild-type mice (solid columns) and AhR-KO mice (dark-shaded columns) repopulated with wild-type BM cells; **(left)** Peripheral blood, bone marrow (BM) and spleen weight (SPL). #: Vertical axis "parameter" indicate the counts of peripheral red blood cells (RBCs, $\times10^8$/ml) and white blood cells (WBCs, $\times10^6$/ml), BM cellularity ($\times10^5$/femur), and weight of the spleen (mg). **(right)** Numbers of *in vitro* granulocyte macrophage CFUs (CFU-GM, ×500/femur) in femoral BM.

Figure 1B shows the same parameters in the wild-type recipients, repopulated with wild-type (solid columns) or AhR-KO BM cells (light-shaded columns). In the wild-type mice repopulated with AhR-KO BM cells, despite the repopulation with AhR-KO BM, they did not show patterns in the blood parameters equivalent to those shown in **Figure 1A**, implying that the effect derived from the $AhR^{+/+}$ wild-type host cannot be ruled out (see later **Figure 3** results and discussion).

In **Figure 1C**, lethally irradiated wild-type and AhR-KO mice were repopulated with the same wild-type BM cells. Possibly because of the same reasons discussed in the case of **Figure 1A**, the number of RBCs of the AhR-KO mice repopulated with wild-type BM cells showed a significant decrease, and similarly, those mice showed a significant increase in splenic weight. Concerning the CFU-GM, the decrease in number observed in the AhR-KO mice repopulated with wild-type BM cells clearly showed an increase to the level of the control, i.e., lethally irradiated wild-type mice repopulated with wild-type BM cells. In this regard, the hematopoietic functions of the BM were recovered upon the repopulation with wild-type BM cells; however, some functional deficiencies in the AhR-KO mice cannot be recovered in the peripheral blood despite the repopulation with wild-type BM cells.

Benzene-induced hematopoietic impairment in the hematopoietic progenitor level (CFU-GM) is attenuated in the wild-type mice repopulated with AhR-KO BM cells. As mentioned in an introduction, the benzene-induced hematopoietic impairment in the progenitor cell level was attenuated in the AhR-KO mice[1]. Also, lethally irradiated wild-type mice repopulated with BM cells from AhR-KO mice showed an attenuation of benzene-induced hematopoietic impairment in the progenitor cell level[2]. Namely, the benzene-induced decrease in the number of CFU-GM in the BM of mice repopulated with the AhR-KO BM cells was clearly nullified for the wild-type BM cells, and the number stays within the range found for the sham exposure (data not shown).

Benzene-induced hematopoietic impairment in the hematopoietic progenitor level (CFU-GM) is reappeared in the AhR-KO mice repopulated with wild-type BM cells. Next, the AhR-KO mice were repopulated with wild-type BM cells. Namely, a question in this study is to see whether the benzene-induced hematopoietic impairment in the level of progenitor cells would be observed in the lethally irradiated AhR-KO mice repopulated with wild-type BM cells.

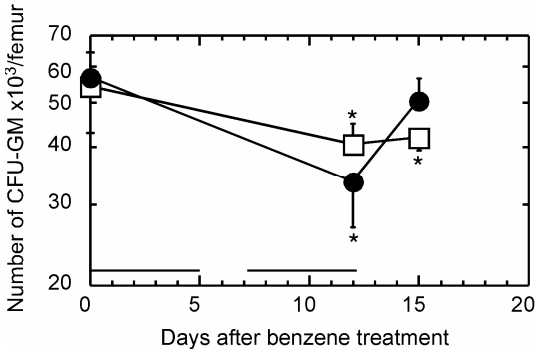

Figure 2: Changes in number of CFU-GM/femur of wild-type (open symbols) and AhR-KO (solid symbols) mice repopulated with wild-type BM cells during and after benzene exposure. Vertical bars indicate the standard deviation of the mean. The dashed line at the bottom indicates the benzene exposure duration.
*: Significant difference between sham exposure group and benzene-exposed group determined by *t*-test at p<0.05.

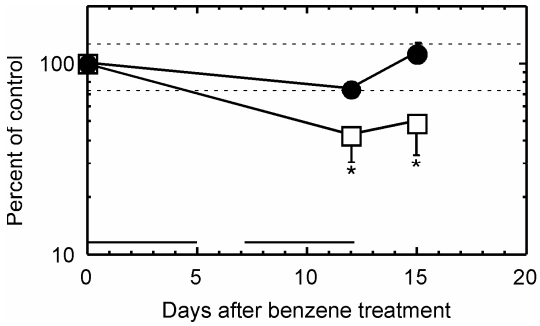

Figure 3: Changes in percentage of WBC of wild-type (open symbols) and AhR-KO (solid symbols) mice repopulated with wild-type BM cells during and after benzene exposure, with respect to each sham exposure group. Vertical bars indicate the standard deviation of the mean. Horizontal dashed lines indicate the standard deviation of the mean (100%) from the sham control wild-type group. The dashed line at the bottom indicates the benzene exposure duration.
*: Significant difference between sham-exposure group and benzene-exposed group determined by *t*-test at p<0.05.

The results are shown in **Figure 2**. The number of CFU-GMs in the lethally irradiated wild-type or AhR-KO mice repopulated with wild-type BM cells shows a similar decrease 12 days after the benzene exposure, and a similar recovery was observed within 3 days. In this regard, again, the impairment of hematopoietic progenitor cells in the BM after benzene exposure is dependent on the hematopoietic progenitor cells themselves with their own simultaneous expressions of AhR and CYP2E1.

Benzene-induced hematopoietic impairment in the peripheral blood may be attributable to the hepatic AhR-mediated toxic metabolism. As observed in **Figure 1C** and compared with **Figure 1A**, $AhR^{+/+}$ wild-type, other than BM-AhR, was seemingly responsible for the hematopoiesis of the peripheral blood; to determine whether such wild-type AhR other than BM, possibly hepatic AhR, might play a role in the benzene-induced hematopoietic impairment or the level of peripheral blood, the number of WBCs was precisely compared between lethally irradiated wild-type (open symbols) and AhR-KO (solid symbols) mice, both repopulated with wild-type BM cells (**Figure 3**). Interestingly, the number of WBCs in wild-type mice repopulated with wild-type BM cells was kept significantly lower than those of the AhR-KO mice repopulated with wild-type BM cells throughout the benzene exposure period. In this regard, the significant decrease in the number of WBCs observed in previous studies[2] as well as in similar reports is presumably based not on the BM-AhR but on the AhR possibly derived from hepatic tissue or other visceral organs.

Conclusions

The benzene-induced hematopoietic impairment was primarily found to be dependent on the AhR in the hematopoietic progenitor cells of the BM; however, interestingly, the benzene-induced hematopoietic impairment in the peripheral blood was found to be dependent on the AhR other than in the BM, possibly in the hepatic tissue; thus, the benzene-mediated toxicity in peripheral blood was attenuated in the lethally irradiated AhR-KO mice repopulated with wild-type BM cells, although the benzene-mediated toxicity in the hematopoietic progenitor cells was clearly reappeared.

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