BENZENE-INDUCED HEMATOPOIETIC TOXICITY TRANSMITTED BY AHR IN THE WILD-TYPE MOUSE WAS NEGATED BY REPOPULATION OF AHR DEFICIENT BONE MARROW CELLS.

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Introduction

Recent studies have shown that the aryl hydrocarbon receptor (AhR) in primitive cells transmits negative signals for the proliferation of such cells[1, 2]. As we previously reported, primitive hemopoietic progenitor cells increases in number in AhR-knockout (KO) mice; on the other hand, relatively mature progenitor cells on the other hand, decreases in number in a homeostatic manner[1].

We have reported that benzene-induced hemopoietic toxicity is transmitted by AhR[3]. We also found that cytochrome P450 2E1 (CYP2E1) related to benzene metabolism is also up regulated in the bone marrow by benzene exposure in the bone marrow[4]. Therefore, it is of interest to hypothesize a greater role of bone marrow cells in hemopoietic toxicities rather than the hepatic metabolism. Accordingly, in the present study, benzene-induced hemopoietic toxicity was evaluated in wild type (Wt) mice after a lethal dose of whole-body irradiation followed by repopulation of bone marrow cells that lack AhR or, *vice versa,* in AhR KO mice after repopulation of Wt bone marrow cells.

As results, benzene-induced hemopoietic toxicity seems to have been transmitted through AhR, and benzene was transformed by *de novo* metabolism with CYP2E1 in the bone marrow.

Materials and Methods

Animals. The establishment of homozygous AhR KO ($AhR^{-/-}$) mice, the 129/SvJ strain, is described elsewhere[3, 5]. 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 repopulation assay and the assay of CFU 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 (PLT) counts were determined using a blood cell counter (Sysmex M-2000, Sysmex Co., Kobe, Japan). Bone marrow (BM) cellularity was evaluated by harvesting BM cells from the femurs of each mouse[6]. 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 a-MEM. A single-cell suspension was obtained by gently triturating the BM cells through the 27-gauge needle, and cells were counted using Sysmex M-2000.

Irradiation. Recipient mice were exposed to a lethal radiation of 800.1 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[7]was used to determine the number of colony-forming units 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 after the injection, and fixed in Bouin's solution. Macroscopic spleen colonies were counted under an inversion microscope at a magnification of x 5.6.

CFU-GM and CFU-E Assay. *in vitro* colony formation was assayed in semisolid methylcellulose culture[6, 8]. Briefly, 8 x 10^4 BM cells suspended in 100 ml 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 erythro-poietin for erythroid CFU (CFU-E). One-ml aliquots containing 2 x 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 x 40 for CFU-GM after 6-day culture and x100 for CFU-E after 3-day culture.

***BM repopulation assay*[9].** The BM repopulation assay was performed similarly to the assay of CFU-S, except that 10^6 BM cells were injected into lethally irradiated mice. One month after the transfusion of BM cells, the repopulated mice were used in the experiment.

Results and Discussion

As previously reported, AhR-KO mouse showed a significant increase in WBC counts (**Figure 1** A). This was also consistent with the high number of myeloid progenitor cells, *i.e.*, CFU-S-9 and CFU-S-13, observed in the AhR-KO mice (**Figure 1**, B). Thus, steady-state hemopoiesis is presumed to be suppressed by AhR signaling due to the possible presence of a physiological ligand, which is not readily observed in AhR-KO mice. In response to such an AhR-null effect, the AhR-KO mouse reversely shows extensive hemopoiesis in the spleen, although this hemopoietic enhancement is also reflected in another negative hemopoietic regulation in the BM. Accordingly, in the present study, benzene-induced hematotoxicity was evaluated in the Wt mice after a lethal dose of whole-body irradiation followed by the repopulation of BM cells that lack AhR.

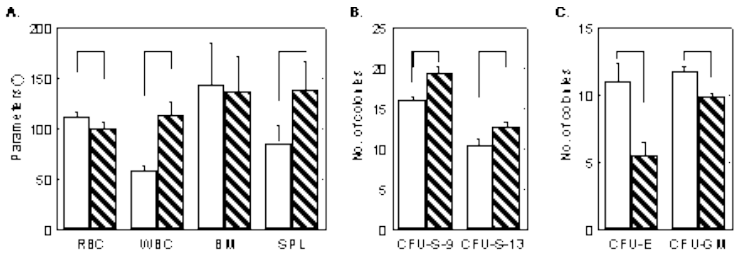


Figure 1: Comparison of various blood parameters between Wt mice (open columns) and AhR-KO mice (shaded columns) [1]; **A**. Peripheral blood, bone marrow and spleen weight. * Parameters indicate the counts of peripheral red blood cells (RBCs, $\times 10^8$/ml) and white blood cells (WBCs, $\times 10^6$/ml), bone marrow cellularity (BM, $\times 10^5$/femur), and weight of spleen (SPL, mg). **B**. Number of colony-forming units 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 CFUs (CFU-GM/5×10^3 BM cells) and erythroid CFU (CFU-E/1×10^4 BM cells). †: Significant difference between Wt and AhR-KO mice determined by *t*-test at $p<0.05$.

Figures 2, A-C, show the RBC (A), WBC (B), and platelet (PLT: C) counts (per mL) in the peripheral blood after repopulation of the BM. In each figure, in the Wt mice repopulated with Wt BM cells (two columns on the left), the groups subjected to intraperitoneal benzene exposure (second from the left) show significant decreases in RBC and PLT counts (92% and 69%; p=0.010 and 0.016, respectively) compared with the sham exposure groups (farthest left in each figure), except 2B, i.e., WBC counts (96%). When the mice repopulated with AhR-KO BM cells (two columns on the right) are exposed to benzene, there are no significant differences between the sham exposure groups (second from the right) and the benzene exposed groups (farthest right) in A through C. Significant decreases

observed in the Wt mice repopulated with Wt BM cells were negated when the Wt mice were repopulated with AhR-KO BM cells; thus, the reduction in the number of peripheral blood cells observed in the Wt mice after benzene exposure is assumed to be responsible for the AhR expression in BM cells. Although the two sham exposures (i.e., Wt mice, open column; and AhR-KO mice, solid column) are essentially identical in A and C, there seems to be insufficient recovery of the BM in transplantation in Figure 2B, and the solid column is significantly reduced (see, Figure 3 on CFU-GM).

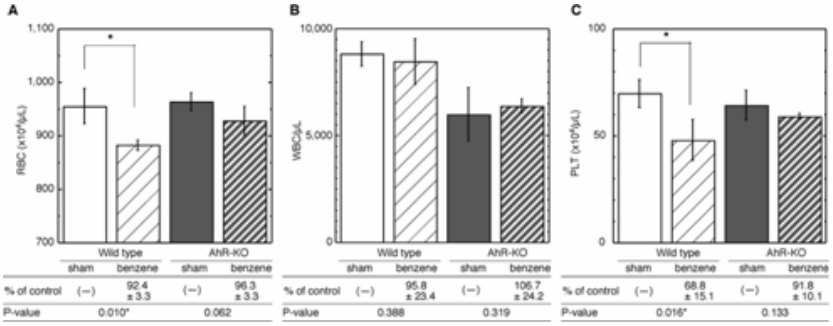


Figure 2: Comparison of various blood parameters in peripheral blood; A, RBC, B, WBC, and C, platelets (open bars vs lightly shaded bars in Wt mice repopulated with Wt BM cells; solid bars vs heavily shaded bars in Wt mice repopulated with AhR BM cells).

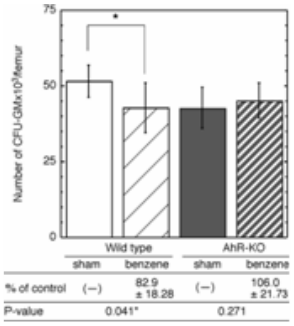
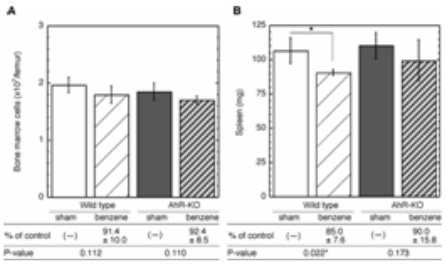


Figure 3: *: Significant difference between sham and exposed determined by *t*-test at $p<0.05$.

In Figure 3, again, the significant decrease in the number of granulocyte-macrophage colony-forming units *in vitro* (CFU- GM/5 x10^3 BM cells) in the BM cells from the Wt mice repopulated with Wt BM cells (82.9% in benzene exposure, lightly shaded column to the right of the sham exposure, open leftmost column; p=0.041) is negated in the BM cells from mice repopulated with AhR-KO BM cells (sham exposure, solid column; and benzene exposure, heavily shaded column, respectively). In this figure, the efficiency of repopulation with AhR- KO BM cells (solid column) seems to be insufficient, since the solid column is smaller than the open column (p=0.025). The mechanism underling the incomplete recovery of AhR-KO BM cells, is still unknown; however, the sublethal irradiation of the recipient mice may be the case, where suppressive intrinsic factors may have been released from tissues given the lethal dose of irradiation received by the host animals.

Despite the insufficient recovery of the number of GM-CFU in mice repopulated with AhR-KO BM cells, number of BM cells, regardless of repopulated cell type (either Wt or AhR-KO BM cells) and type of exposure (either benzene or sham exposure), there were no significant differences in number of BM cells among the groups in a homeostatic manner (**Figure 4**, A; 91.4% and 92.4%, respectively, p>0.1). However, after benzene exposure, a significant decrease in splenic weight was observed in the Wt→Wt group (85.0%, p=0.022), but not in the AhR-KO-BM→Wt group (90.0%, p=0.173). This supports the notion that AhR-KO negates the suppressive effect on splenic weight after benzene exposure.

Figure 4: Comparison of number of BM cells (A) or weight of spleen (B) with or without benzene exposure, in mice repopulated with Wt BM cells or in mice repopulated with AhR-KO BM cells. (Wt mice repopulated with Wt BM cells, lightly shaded columns, second from left; or without benzene exposure, open columns, farthest left, benzene exposure vs Wt mice repopulated with AhR-KO BM cells, each of the two right columns).



Conclusions

The up-regulation of CYP2E1 after benzene exposure was specifically observed in our previous microarray study of the bone marrow tissue[4]. The analysis of the gene expression specifically derived from the hematopoietic stem cell compartment[10], and the evaluation of the toxicological alteration of such an expression as a measure of stem cell specific toxicological biomarkers are hot issues in the current hematotoxicology[11]. Mice that have been lethally irradiated and repopulated with BM cells from AhR-KO mice essentially did not shown any benzene-induced hematotoxicity, implying that such toxicity is derived from *de novo* metabolisms with CYP2E1 in the BM other than hepatic metabolism. The present study raises two questions on AhR-mediated TCDD-induced hematotoxicity: Do Wt mice repopulated with AhR-KO BM cells show hematotoxicity by TCDD unlike in the case of benzene exposure? If such is the case, what would be the transmitter from the site of xenobiotic metabolic activation to the bone marrow?

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