Chemico-Biological Interactions xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Benzene-induced bone-marrow toxicity: A hematopoietic stem-cell-specific, aryl hydrocarbon receptor-mediated adverse effect

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ARTICLE INFO

Article history:
Available online xxx

Keywords:
Hematopoietic stem cells
Hematopoietic progenitor cells
Global gene-expression profiles
Common gene expressions
Stochastic signalings

ABSTRACT

Benzene-induced hematopoietic toxicity is an aryl hydrocarbon receptor (AhR)-related adverse effect that is not exhibited in AhR-knockout (KO) mice. In the hematopoietic system, the steady-state expression of AhRs is limited in the hematopoietic progenitor cells; thus, a hierarchical hematopoietic impairment starts from hematopoietic progenitor cells after benzene exposure. When one looks at wild-type recipient mice that have been lethally irradiated and repopulated with AhR-KO bone marrow cells, owing to reconstruction by the marrow from AhR-KO mice, no impairment is observed in the assay of granulo-macrophage colony-forming units (CFU-GMs) in the bone marrow after benzene exposure of the reconstituted mice. In contrast, in mature white blood cells concern, benzene-induced hematopoietic cytotoxicity is observed in the same reconstituted mice; however, this benzene-induced hematopoietic cytotoxicity in mature white blood cells is not induced in the case of AhR-KO mice repopulated with wild-type bone marrow cells after a lethal dose of irradiation. The mechanism of benzene-induced hematopoietic toxicity in the mature blood cells in AhR-KO mice is assumed to be based on metabolites such as phenol and hydroquinone derived from hepatic AhR. Thus, the former toxicity in mature white blood cells is assumed to be based on the metabolites of the wild-type hepatic AhR, whereas the latter lack of toxicity in mature blood cells in AhR-KO mice is due to the lack of benzene-induced metabolism in the liver. Global gene expression analysis of bone marrow cells after benzene exposure reveals that *MEF2c*, the functions of which are known to maintain lymphocyte differentiation and promote proliferation of hematopoietic progenitor cells, is commonly downmodulated not only in C57BL/6 but also in C3H/He mice. In response to these impairments of the hematopoietic progenitor cells and the niches, stochastic and reciprocal upregulations of *integrin beta 2* and the *Runx* family are observed, which are known to stabilize hematopoietic niches during the steady-state. Direct observation of the hematopoietic progenitor cells, particularly the $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ (LKS) fraction, after benzene exposure revealed an increased amount of intracytoplasmic reactive oxygen species (ROS) detected by ROS-reacting dye as compared with other blood cell fractions.

1. Introduction

All peripheral blood cells in circulation, including circulating "stem cells", in aryl hydrocarbon receptor (AhR)-knockout (KO) mice do not exhibit benzene-induced hematopoietic cytotoxicity due to a lack of AhR in either the liver or in the bone marrow [1], even if the KO mice are repopulated with wild-type bone marrow cells [2]. However, as reported previously, hematopoietic progenitor cells in the bone marrow solely exhibit severe toxicity when KO mice are repopulated with AhR^{+} wild-type bone marrow cells [2]. Experimental results above were implied that the toxicity observed solely in the hematopoietic progenitor cells was owing to transplanted bone marrow cells carrying AhR-related to cell-cycle-perturbation [3], yet the relationship between benzene-induced signaling pathway and the cytochrome P450 (CYP) 2E1 metabolism is not fully elucidated. This AhR-mediated cell-cycle-perturbation in the hematopoietic progenitor cells can be called as a "cell-cycle-mediated hematotoxicity", and this is observed solely in the bone marrow. In contrast, the mechanism of benzene-induced hematotoxicity in all blood cells in circulation, including circulating stem cells, is considered to involve metabolites such as phenol and hydroquinone, derived from the hepatic AhR (metabolite-mediated chemical toxicity) [2,4]. This metabolite-mediated hematotoxicity

Abbreviations: AhR, aryl hydrocarbon receptor; CFU-GMs, granulo-macrophage colony-forming units; CFU-Ss, colony-forming units in spleen; DCFH-DA dye, 2′,7′-dichlorodihydrofluorescein diacetate dye; KO, knockout; LKS cell, $lineage^{-}$ c-Kit+Sca-1+ cell; ROS, reactive oxygen species; WBCs, white blood cells.

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doi:10.1016/j.cbi.2009.12.022

is known not to be observed in AhR-KO mice, even if the AhR-KO mice were lethally irradiated and repopulated with wild-type bone marrow cells [2].

The above-mentioned mechanism of action suggests that the benzene-induced bone-marrow toxicity in the former is a hematopoietic stem-cell-specific, AhR-mediated adverse effect; thus, in general, it involves an extremely low-dose range, theoretically, whereas the latter hepatic (and other tissue derived) metabolite-mediated chemical toxicity for all blood cells, including circulating stem cells may exhibit a threshold.

Global gene expression of bone marrow cells after benzene exposure analyzed previously revealed detailed underlying xenobiotic responses of the bone marrow [5,6]. In the present studies, as a common expressing gene profiling related to hematopoiesis and hematopoietic niches, the expression of *integrin alpha 4*, the function of which is known to be maintaining the pluripotency, stemness, of hematopoietic progenitor cells [7] as well as maintaining cell-cycle dormancy [8], was downregulated and, thus, hematopoietic niches were assumed to be commonly impaired. *MEF2c*, the functions of which are known to maintain lymphocyte differentiation [9] and promote proliferation of pluripotent hematopoietic progenitor cells [10,11], was downregulated not only in C57BL/6 but also in C3H/He mice. In response to these impairments of the hematopoietic progenitor cells and the niches, stochastic and reciprocal upregulations of *integrin beta 2* and the *Runx* family, which is known to function to stabilize hematopoietic niches [12,13], were observed. Interestingly, concomitant stochastic gene alterations, such as *VCAM1*, *cadherin-11* and *B-cell leukemia/lymphoma 6* (*Bcl-6*), were also stochastically modulated for plausible epigenetic leukemogenic signals [14–17].

Direct observation of the hematopoietic progenitor cells, particularly the $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ (LKS) fraction, after benzene exposure revealed an increased amount of intracytoplasmic reactive oxygen species (ROS) detected by ROS-reacting dye as compared with the fraction without benzene exposure. Prolonged ROS was detected in the LKS by ROS-reacting dye 28 days after the last benzene exposure.

In the present study, benzene-induced hematotoxicities were started from two bilateral aspects; in the case of hematopoietic progenitor cells, the impairment was started from the induction of oxidative stress and an increased amount of intracytoplasmic ROS in LKS stem cells, followed by consequent blood cell toxicity with their differentiation and maturation, whereas in the case of mature hematopoietic cells, toxicity was induced by AhR-mediated drug metabolism in nonhematopoietic tissues specifically in the hepatic tissue.

2. Materials and methods

2.1. Animals

The establishment of homozygous AhR-KO ($AhR^{-/-}$) mice originating from the 129/SvJ strain has been described elsewhere [1,18]. The crossing of males and females of heterozygous AhR-KO ($AhR^{+/-}$) which have been backcrossing with C57BL/6CrSlc over 20 generations in National Institute of Health Sciences (NIHS), Japan, generated wild-type ($AhR^{+/+}$), $AhR^{+/-}$, and $AhR^{-/-}$ mice. The neonates were genotyped by PCR screening of DNA from the tail. AhR-KO ($AhR^{-/-}$) mice and their wild-type littermates were used in the study. C57BL/6 mice from Japan SLC (Shizuoka, Japan) were used for microarray study, oxidative stress status assay and also as recipients for the repopulation assay and the assay of colony-forming unit in the spleen (CFU-S). For microarray study, C3H/He mice from Japan SLC were also used. All the mice were housed under specific pathogen-free conditions at $24 \pm 1\,^{\circ}C$ and $55 \pm 10\%$ relative humidity, under a 12-h light–dark cycle. Autoclaved tap water and food pellets were provided *ad libitum*.

All the animals were maintained in a board-approved laboratory animal facility at NIHS, Japan. All experimental protocols involving the laboratory mice used in this study were reviewed by the Interdisciplinary Monitoring Committee for Proper Animal Use and Welfare of Experimental Animals (ICRAW), a peer review panel established at NIHS, and approved by the Committee for Animal Care and Use of the NIHS (CACU) with the experimental code #109-2007. All animal studies were conducted using humane protocols approved by the Committee for Animal Care and Use of the NIHS, Japan.

2.2. 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 treated with benzene by inhalation (300 ppm, 6 h/day for 5 days/week for 2 weeks) or intragastrically (*i.g.*) administered with freshly prepared corn oil solutions of benzene (150 mg/kg body weight (b.w.), once daily for 5 days/week for 2 weeks). Both doses administered for 5 days/week for 26 weeks induce hematopoietic malignancies at the highest frequency during the lifetime [19–23]. Detailed inhalation procedure including dose monitoring for benzene exposure was described elsewhere [1,3,5,20,21]. The aim of this study using this dose is to examine the corresponding toxicity of benzene for inducing hematopoietic malignancies. Note, this dose is over 100-fold higher than the occupational tolerable exposure dose.

2.3. Blood and bone marrow parameters

Peripheral blood was collected from the orbital sinus and bone marrow cells were harvested from the femurs of each mouse [3]. Peripheral blood leukocyte (WBC), red blood cell (RBC) and platelet (PLT) and also a single-cell suspension of bone marrow cells were counted using a blood cell counter (Sysmex K-4500, Sysmex Co., Kobe, Japan).

2.4. Antibodies and immunomagnetic bead separation

For the depletion of differentiated (lineage marker positive) cells from bone marrow cells, immunomagnetic bead separation (BD IMag Mouse Hematopoietic Progenitor Cell Enrichment™ set (BD Biosciences, San Jose, CA)) was performed followed by the manufactured procedure. For lineage (Lin) markers, a biotinylated antibody cocktail (BD Biosciences) containing anti-mouse CD3e (145-2C11), CD11b (M1/70), CD45R/B220 (RA3-6B2), Ly-6G and Ly-6C/Gr-1 (RB6-8C5), and TER-119/erythroid cell (TER-119) antibodies was used. As a secondary antibody for the former biotinylated antibody cocktail, streptavidin (StAv)-coated beads (BD Biosciences) for depletion and StAv–peridinin chlorophyll-a protein (PerCP, BD Biosciences) for visualization were used.

Bone marrow cells depleted of differentiated cells (ca. 1% of unfractionated bone marrow cells) were stained with antibodies for CD117/c-kit, conjugated with allophycocyanin (APC, BD Biosciences) and for stem cell antigen (Sca-1), conjugated with phycoerythrin (PE, BD Biosciences) to determine LKS fraction as a lineage marker for differentiation-negative, c-kit-positive, and Sca-1-positive fraction.

2.5. Irradiation

Recipient mice, AhR-KO mice or wild-type mice for repopulation assay or wild-type mice for the assay of CFU-S, were exposed to a lethal-dose radiation of 801.2 cGy, at a dose rate of 102.5 cGy/min,

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using a ^{137}Cs-gamma irradiator (Gammacell 40 Exactor, MDS Nordin Inc., Canada) with a 0.5 mm aluminum–copper filter.

2.6. Bone marrow repopulation assay

Bone marrow repopulation assay [24] was performed similar to the assay of CFU-S, except that 1×10^6 bone marrow cells were injected into lethally irradiated mice. One month after the transfusion of bone marrow cells, the repopulated mice were exposed to benzene.

2.7. Assay for hematopoietic progenitor cells

For determining the number of CFU-S, the Till and McCulloch method [25] was used. Colony formation *in vitro* was assayed in a semisolid methylcellulose culture with 10 ng/ml murine granulocyte–macrophage colony-stimulating factor (GM-CSF) for assay of granulo-macrophage colony-forming units (CFU-GMs) in the bone marrow [3,26].

2.8. BUUV assay

Hematopoietic progenitor cell-specific kinetics were evaluated by continuous labeling by an osmotic mini-pump (Alza Corp., Palo Alto, CA) of bromodeoxyuridine (BrdUrd) for cycling cells, followed by ultraviolet A (UVA) exposure and hematopoietic colonization assay (BUUV assay; details were described in elsewhere [26,27]).

2.9. Measurement of ROS production in LKS and/or unfractionated bone marrow cells after benzene treatment

In the assessment of ROS production, unfractionated bone marrow cells or LKS fraction from mice, 24 h after a single benzene treatment (150 mg/kg b.w., *i.g.*) or 28 days after the last benzene treatment (150 mg/kg b.w., *i.g.*), once a day, 5 days a week, for 2 weeks, and those from sham controls were analyzed their intracellular fluorescence intensity by flow cytometry with a 25 μM fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma, St. Louis, MO). Detailed methods were described elsewhere [20].

2.10. Analysis of mRNA expression level by microarray

Total RNA was extracted from bone marrow cells collected from each individual mouse at 28 days after the last benzene *i.g.* treatment, both C57BL/6 and C3H/He, with or without 2 weeks benzene exposure (150 mg/kg b.w., *i.g.*, once a day, 5 days a week), and applied on a GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) containing 45,101 probe sets, as described elsewhere [5,28].

2.11. Statistical analysis

The obtained microarray data were normalized and analyzed using GeneSpring GX 7.3.1 (Agilent Technologies Inc., Santa Clara, CA), SPSS® 14.1 (SPSS Inc., Chicago, IL) and Microsoft Office Excel 2003 (Microsoft, Redmond, WA). For common gene expressions, Welch-*t*-test for each strain separately with or without benzene exposure or two-way analysis of variance (ANOVA) based on the two strains, C57BL/6 and C3H/He, with or without benzene exposure was applied. For stochastic gene expressions, principal component analysis (PCA) was applied to maintain the stochasticity of expression characteristics. The gene-expression profiles of the vehicle control of each strain were compared separately with each individual expression profile of benzene-exposed mice by PCA followed by the selection of genes with high contribution scores from certain PCA-components. Two separate union gene lists for each strain can be generated from each PCA combination corresponding to individual mice exposed benzene.

3. Results and discussion

3.1. AhR-mediated, benzene-induced differential toxicities in hematopoietic stem/progenitor cells and circulating blood cells

Benzene-induced hematotoxicity is an AhR-mediated adverse effect that is not exhibited in AhR-KO mice [1]. Therefore, after benzene exposure, AhR-KO mice show no decrease in the number of white blood cells (WBCs) compared with that in wild-type mice [1,2,4]. However, in the hematopoietic system, the steady-state expression of AhRs is only limited in the hematopoietic progenitor cells; thus, a hierarchical hematopoietic impairment starts from hematopoietic progenitor cells after benzene exposure. These hematopoietic progenitor cells are known to possess CYP2E1 concomitantly [29,30], which is considered to induce stem-cell-limited oxidative stress and consequent cell-cycle arrest due to the upregulation of $p21^{waf1}$ via *Trp53*, resulting in a decrease in the number of mature blood cells in the bone marrow [3]. In fact, in the case that AhR$^+$ bone marrow cells were depleted by lethal-dose radiation, followed by repopulation of AhR-KO bone marrow cells, no toxicity was observed in the assay of granulo-macrophage colony-forming units (CFU-GMs) in the bone marrow after benzene exposure of the reconstituted mice [4]. Benzene-induced hematopoietic stem-cell toxicity had been speculated since Cronkite et al. [31] observed a prominent decrease in the number of colony-forming units in spleen (CFU-S) in the bone marrow after benzene inhalation. Because hematopoietic progenitor cells, particularly the LKS fraction, were found to express AhR, whereas unfractionated bone marrow cells exhibited no detectable AhR expression [32], the present observation is the first evidence suggesting that the benzene-induced target of the hematopoietic system is hematopoietic progenitor cells, particularly the LKS fraction.

In contrast, in circulating mature WBCs, benzene-induced hematopoietic cytotoxicity was not negated, but was observed in the same reconstituted mice with AhR-KO bone marrow [4]; furthermore, this benzene-induced hematopoietic cytotoxicity in mature WBCs was not induced in the case of AhR-KO mice repopulated with wild-type bone marrow cells after a lethal dose of irradiation [2]. From the above experimental results, the finding that mature WBCs, regardless of whether they were derived from AhR-positive or AhR-negative bone marrow cells, were decreased in number in hepatic tissue (and possibly other drug-metabolizing organs) with AhRs, implied that the toxicity of circulating mature blood cells was based on benzene metabolites, resulting from hepatic AhR-related metabolism after benzene exposure, such as phenol and hydroquinone. The mechanisms of benzene-induced hematopoietic toxicities are thus categorized into two: first, AhR (in hematopoietic progenitor cells)-mediated, cell-cycle arrest-induced hematopoietic impairment and, second, hepatic AhR-related metabolite-induced cytotoxicity after benzene exposure. A model is shown in Fig. 1. The former involves an extremely low-dose effect, in general, owing to its mechanism linked to receptor-mediated toxicity; whereas the latter involves metabolite-mediated chemical toxicity with a possible threshold, although this requires further study.

3.2. Impairment of immature hematopoiesis in bone marrow by benzene exposure analyzed by global gene expressions

In the steady-state bone marrow, hematopoietic progenitor cells are assumed to be in the phase of extremely slow cell cycle, as

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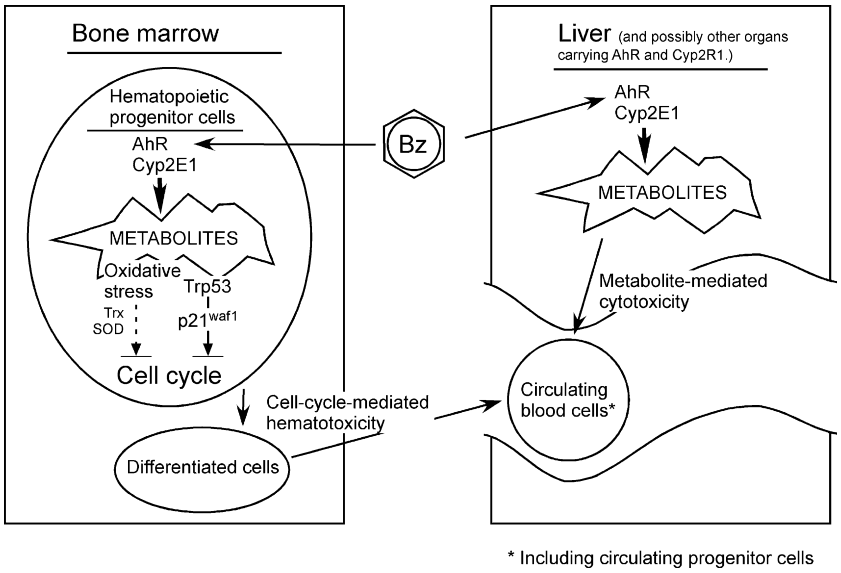

Fig. 1. Differential toxicities: hematopoietic progenitor cells of blood in the bone marrow (left panel) and mature blood cells in the liver (right panel). Both toxicities are transmitted by aryl hydrocarbon receptors. The former is based on cell-cycle arrest-related hematotoxicities and the latter is based on metabolite-mediated cytotoxicity. See text.

previously refer to dormant stem cells. This concept is derived from the fact that the numbers of CFU-Ss, on both days 9 and 13, from AhR-KO mice were higher than those of wild-type CFU-Ss [33]; thus, a function of AhR may contribute to the restoration of the stem cell compartments quiescent in wild-type mice. In fact, the direct measurement of cell kinetics on the hematopoietic progenitor cells, both CFU-S-9 and -13, reveals that the dormant fractions are larger in both progenitor cells [33], as measured by the BUUV method [26,27], a tool to measure cell kinetics specifically for hematopoietic stem cells.

As mentioned above, hematopoietic progenitor cells are recently assumed to be in an extremely low in cell cycle owing to their nearly continuous incorporation of BrdUrd [34]. Furthermore, a physiological and equivalent low level of oxidative stress may accelerate the cell cycle in the hematopoietic progenitor cells, presumably by down-modulation of AhR, in association with p53-mediated-paradoxical downregulation of thioredoxin [35]. However, far higher oxidative stresses, ROS induced by xenobiotics such as benzene exposure, induce cell-cycle arrest owing to toxicological cellular damages [3].

To elucidate the underlying mechanisms of the alteration of hematopoietic progenitor cells and hematopoietic progenitor niches with and without benzene exposure, global gene-expression profiles of the bone marrow were obtained by microarray analysis to observe alterations in gene expression. Gene-expression profiles from two different mouse strains with and without benzene exposure after 28 days reveal two conceptually different categories, one for common gene-expression profiles and the other for stochastic ones; the former repertory is common from one individual mouse to another, the latter repertory is stochastic among individual mice in each group. As a common finding of gene profiling related to hematopoiesis and hematopoietic niches, the expression of *integrin alpha 4*, the function of which is maintaining the stemness of the hematopoietic progenitor cells [7] as well as maintaining cell-cycle dormancy [8], is downregulated and, thus, hematopoietic niches are assumed to be commonly impaired. A sample common gene, *MEF2c*, the functions of which are to maintain lymphocyte differentiation [9] and promote proliferation of pluripotent hematopoietic progenitor cells [10,11], is, on average, downregulated not only in C57BL/6 but also in C3H/He mice. *MEF2c* was found to receive three reciprocal signalings from neighboring genes expressed to various extents, as shown in Fig. 2. Among these three reciprocal signalings, the *retinoblastoma* (*Rb*)-mediated *PCAF* (p300/cyclic AMP-responsive element binding protein-binding protein [CBP]-associated factor)-signal enhances cellular differentiation [36,37], and the *TGF-beta*-mediated *TGF-beta* receptor signals inhibit cellular differentiation through *Smad3* [38]; thus, these two signals reciprocally modulate *MEF2c*'s transcriptional activity, i.e., so-called "choice" of differentiation for either lymphoid lineage or myeloid lineage [11], stochastically, during the steady-state. Following benzene exposure, in contrast, due not only to the *Rb* pathway but also to another pathway from *Eid1* (E1A-like inhibitor of differentiation 1) [39], *PCAF* function is mostly downregulated; therefore, *MEF2c* is downregulated and its transcriptional activity is decreased, thus, myeloid differentiation by bilateral regulation has been consequently chosen after benzene exposure. In addition, note that reciprocal histon deacethylase against *PCAF* relatively strongly inhibits *MEF2c*, as well [40]. These reciprocal regulations by stochastic gene expressions for transcriptional activity of *MEF2c*, i.e., common gene expression, may be based on the various epigenetic changes, of which detailed underlying mechanism require confirmation by increased number of epigenetic data clusterization.

In Fig. 2, gene expressions in mice are designated with expression intensities with light to dark color in boxes separately from C57BL/6 mouse #1 (01), through #5 (05) in the upper row, and from C3H/He mouse #6 (06) through #10 (10) in the lower row. Expression intensities in boxes for C57BL/6 shown in the upper row and those for C3H/He in the lower row vary significantly in each stochastic gene. Consequently, in this benzene exposure case, *MEF2c* signals in both strains are shifted to myeloid differentiation using different pathways. In response to these impairments of immature hematopoiesis and the hematopoietic niche, downregulation of *Gna13* (guanine nucleotide-binding protein alpha 13, which is downregulated by *Runx2*, and promotes osteoblastic proliferation [41]) in C57BL/6 after benzene exposure seems to activate the reciprocal expression of *integrin beta 2* [42], which stabilizes the niche, i.e., in good agreement with the upregulation of *Runx* to maintain hematopoiesis [12].

Concomitant stochastic gene alteration for plausible epigenetic leukemogenicity may be suggested by an upregulation of *VCAM1* associated with *Hif1*-upregulation, a downregulation of *E-cadherin*

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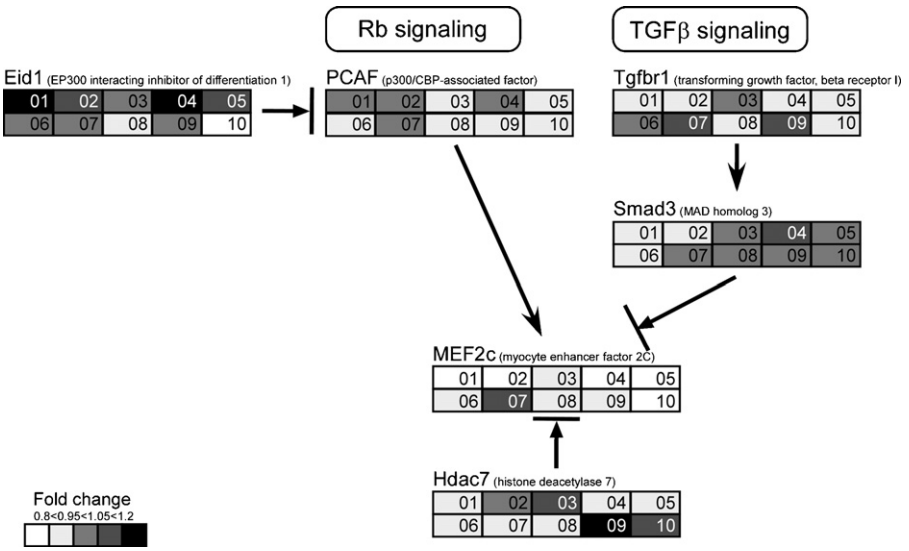

Fig. 2. Gene expressions in mice are designated with expression intensities with light to dark color in boxes separately from C57BL/6 mouse #1 (01), through #5 (05) in the upper row and from C3H/He mouse #6 (06) through #10 (10) in the lower row. *MEF2c*, a promoter of proliferation of pluripotent hematopoietic progenitor cells, is a common gene, which is, on average, downregulated after benzene exposure. The *MEF2c* signal is stochastically regulated by three signaling pathways from stochastic expression genes; the first from *Eid1* via *PCAF*, the second from *Tgfbr1* via *Smad3*, and the third from *Hdac7*. The combinations of these stochastic gene expressions in each mouse are variable and probabilistic, the interrelationships of which lead to reciprocal regulation of the downstream *MEF2c* expression.

induced by the upregulation of *Hif1* through *Zeb1* and *Zeb2* regulation, and also an upregulation of *Bcl-6* [14,15,17,43,44]. In this regard, occasional slight upregulation of the *B-cell-stimulating factor 3* (*Bsf3*)/*cardiotrophin-like cytokine factor 1* (*Clcf1*) in C57BL/6 mice, and common downregulation of that in C3H/He mice, suggest the strain difference of gene expression related to the potential leukemogenicity [45,46].

In conclusion, gene-expression profiling related to the function of hematopoietic progenitor cells and hematopoietic niches has been performed, the results of which suggest the impairment of stem cell niches and consequent proliferation of hematopoietic progenitor cells. These alterations including "very early gene modifications" and "epigenetic leukemogenic plausibility" are obtained unsupervisedly from gene-expression profiles, and the plausibility based on the toxicoinformatics is in good agreement with the above-mentioned cellular kinetics as well as possible epigenetic leukemogenic plausibility.

3.3. *Hematopoietic progenitor cells, LKS fraction in particular, are a possible target of benzene-induced oxidative stress*

Can the effect of benzene-induced ROS in hematopoietic systems be directly evaluated? ROS in unfractionated bone marrow cells as well as in hematopoietic progenitor cells can be evaluated using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) dye. Hematopoietic progenitor cells are quiescent in anoxic environments, and are regulated by a weak oxidative stimulation, such as redox homeostatic regulation [47]. Thus, the reactivity of the fraction to the DCFH-DA dye was higher in AhR-KO mice than in wild-type mice [48], which is in good agreement with the mechanism underlying genomic stabilization under a low oxidative tension in combination with the suppressor gene function and the consequent longevity observed in wild-type mice [48].

To determine whether benzene exposure alters the ROS content of hematopoietic progenitor cells, particularly the LKS fraction, as compared with bone marrow cells, an intermittent benzene exposure (150 mg/kg b.w., *i.g.*, once a day, 5 days a week for 2 weeks) was carried out for five C57BL/6 mice, and the amount of ROS in the bone marrow cells and LKS fraction was evaluated using DCFH-DA dye, one or 28 days after the last exposure.

The amount of ROS in the bone marrow immediately after singly dose of benzene exposure was not high in mice with benzene exposure than in those with vehicle treatment, however in the case of the LKS fraction, ROS-level was apparently lower than that of the steady-state bone marrow, whereas the fluorescence after benzene exposure increased to the level of bone marrow (data not shown). Thus, the changes in ROS in the bone marrow and those in the LKS seemed to be different before and after benzene exposure, however, further studies elucidating detailed dose-response specificity including time courses between those in bone marrow and in the LKS are required for confirmation, because there has been observed some inconsistent data in the whole set of examination.

4. Conclusions

Mechanisms of benzene-induced hematopoietic toxicities are categorized into two: first, a cell-cycle arrest-induced hematopoietic impairment in hematopoietic progenitor cells carrying AhR, and, second, metabolite-induced cytotoxicity related to hepatic AhR, both after benzene exposure. The former involves a low-dose effect, in general, owing to its mechanism linked to receptor-mediated toxicity; whereas the latter involves metabolite-induced xenobiotic chemical toxicity with a possible threshold, although this requires further study.

Global gene-expression profiling related to the function of hematopoietic progenitor cells and hematopoietic niches has been performed, the results of which suggest the impairment of stem cell niches and consequent proliferation of hematopoietic progenitor cells. These alterations including "very early gene modifications" and "epigenetic leukemogenic plausibility" are obtained unsupervisedly from gene-expression profiles, and the plausibility based on the toxicoinformatics are in good agreement with the above-mentioned cellular kinetics as well as possible epigenetic leukemogenic plausibility.

To determine whether benzene exposure alters the ROS content of hematopoietic progenitor cells, particularly the LKS fraction, as compared with bone marrow cells, the amount of ROS was evaluated using DCFH-DA dye after benzene exposure. Results suggested that the benzene-induced intracellular ROS-accumulation started from the LKS fraction, of which cell cycle impairment

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and consequent metabolites extend further impairment to other mature bone marrow cells.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Funding: This work was supported in part by Grants-in-Aid for Scientific Research C, 15510064, 18510066 and 21510074 and also by the Ministry of Health, Labour and Welfare, Japan (MHLW)-Research Fund (H19-Chemistry 003), National Institute of Health Sciences.

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