補助因子などとの相互作用を惹き起こすことなど，つい先頃までの認識を書き換える驚くべき関係が浮かび上がっている．

内分泌機能をもつ新しい器官の発見に加えて，内分泌器官そのものの概念を変える事象も見いだされている．異物受容体と呼ばれているダイオキシン受容体は，エストラジオールが存在しない状態では，P300と名付けられているタンパク分子の助けで転写活性化を担って，女性ホルモン様の作用をもつことが東京大学分子細胞生物学研究所の加藤茂明のグループによって発見された[37]．しかもここでは，エストラジオールがあるときは，この分子は，反対にユビキチン・リガーゼ(Ubiquitin ligase)と呼ばれる複合体を形成し，エストロゲン受容体を壊して，抗女性ホルモン様の役割を発揮する[38]．これは，ホルモン受容体でもない，異物受容体と呼ばれる生体内分子が，種々のホルモン様の作用を時に機能を変化させつつ発揮するということである．内分泌撹乱問題の分子的基盤が，概念的に大きく拡大しているものと考える所以である．内分泌系の拡がりを認識すれば，この発見の示唆する重みに改めて驚かされるであろう．

「生体調節障害の毒性学」を確立するために

内分泌撹乱化学物質問題が取り上げられるきっかけになったことそのものは，ヒトや野生生物の生殖や内分泌機能に関する危惧にあった．やがてその可能性の原点がホルモン作動性の化学物質の低用量での影響にあるものとの認識に近づいた．しかしこれは従来の試験法では有害性が観察されないなど，その背景となるメカニズムがなかなか明らかにならなかった．

すでに見たように，従来の試験法で観察されなかった背景は，この低用量作用が，従来の試験法が対象としていた毒性とは異なった生体障害機構にもとづくものであったためだった．内分泌撹乱化学物質では，生体内分子の壊変や変質などといった構造異常の前に，むしろ曝露影響は，低用量であるがゆえに通常の生体の生理的調節水準下で目に見えない形で微視的機能不全へと進行し，エピジェネティックに，次第に持続的調節不全に陥る生体異物相互作用の調節異常にもとづくのである．これまで注目されてこなかった事柄であり，事実，受容体を介した諸々の影響に焦点は収束しつつあったが，それらがどのように障害に結びつき得るかは，想定にとどまっていた．それは明らかに従来型の，生体分子の酸化や還元，DNAや脂質などの高分子への付加体形成や架橋形成などの化学的修飾，主として生体物質の壊変，変質といった化学反応を基礎とした直接的構造変化とは異なっており，それらに主眼をおいて作られてきた試験法では評価できなかったということであったと考えられる．

この問題の発端の頃，頻度の低い，胎生期のような形態形成期の事象や，小児の生殖器系に限局した内分泌撹乱現象を，稀な確率論的現象と解釈する報告がなされたことがあった．これも以上のような背景と結びついていたものと考えられる．裏返せば，フィードバック機構や“可逆性”の背後で，むしろ低用量曝露にあってはじめて惹き起こされるこの種の毒性は，それまでの毒性試験では検討されず，想定されていなかった．事実，先頃出版されたタイル(R. W. Tyl)らによるビスフェノールAの2世代試験でも，従来の毒性試験にない幅広の用量点をとった試験であったにもかかわらず，何らの影響も認められなかった[39]．タイルらの試験法と，この間工夫を重ねて行われた一連の研究における実験条件との違いには，前者における，持続投与によって惹き起こされるウィンドウ効果の棄却や，被験動物に不応性が導き出されることなどがあるものと推定されるが，この試験法の無力については，背景の解明，両者の相違の可視化のために，胎生期の狭い期間に限局した特異的な遺伝子発現と，投与異物との相互作用の詳細などが明らかにされる必要がある．

低用量における生体異物相互作用の調節障害は，内分泌撹乱化学物質問題を契機として見いだされた，これまでの毒性学の標的に含まれない生体障害性を基礎としたあたらしい概念の毒性現象であ

る．だから従来の毒性学の方法論に加えて，独自に進めるべき研究課題を含んでいる．たとえばこれらの調節障害を原理とした有害性では，通常の調節が生理的範囲から異常状態に移行する過程が，従来の表現型による線引きの困難ないわば振幅の変化のような，境界を含んでいる．そうした境界には直接的な構造異常が伴っていないようであり，これを裏付ける生体分子シグナル機構のより詳細な研究が必要である．こうした事柄は，あたらしい毒性学で求められるこれまでにない課題である．内分泌撹乱化学物質問題によってはじめて見いだされたこのあたらしい生体障害の概念は，当初の想定を超えて，一般論として生体調節障害の全域に及ぶ課題の拡がりを内包している．この点は，米国内分泌学会の内分泌撹乱物質に関する最近発表されたはじめての公式声明の冒頭でも指摘されている[40]．“化学物質の生体調節障害”という課題を対象としたあたらしい毒性学の確立のためには，さらなる概念の構築・整理と，対応する試験法の樹立を一層確かなものとする必要がある．

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Hematopoietic neoplastic diseases develop in C3H/He and C57BL/6 mice after benzene exposure: Strain differences in bone marrow tissue responses observed using microarrays

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ABSTRACT

In this study, *Trp*53-deficient and wild-type mice of both C57BL/6 and C3H/He strains were exposed to benzene (33, 100, and 300 ppm; 6 h/day, 5 days/week for 26 weeks) and then observed for lifetime. As results, first, the incidence of nonthymic lymphomas in C57BL/6 mice and acute myeloid leukemias (AMLs) in C3H/He mice showed linear responses at the lower exposure level in *Trp*53-deficient mice; second, the incidence of thymic lymphomas in C57BL/6 mice and nonthymic lymphomas in C3H/He mice increased without a plateau-like ceiling; thus, the former equivocal induction of hematopoietic neoplasms (HPNs) in the case of low-dose benzene exposure was assumed to be based on the DNA repair potential in wild-type mice, and the latter limited increase in HPNs in the case of high-dose benzene exposure was considered to be due to excessive apoptosis in wild-type mice. Concerning the incidence of AMLs, though a dose of 300 ppm benzene inhalation induced 9% AMLs in wild-type C3H/He mice-AML-prone, it induced AMLs in 38% of *Trp*53-deficient C3H/He mice. Because AMLs were also observed in *Trp*53-deficient mice, including in the C57BL/6 mice, benzene exposure may also be a potent inducer of AMLs in mice with some strain differences. In the present study, to elucidate the hematopoietic stem cell-specific, aryl hydrocarbon-receptor-related low-dose adverse effect, global gene expression in the bone marrow was analyzed at 28 days after 2-week-intermittent exposure to 150 mg/kg b.w. benzene, by gavage, i.e., equivalent to the above inhalation protocol with 300 ppm. We observed two conceptually different gene expression profiles; "common gene profiles" (CGPs) shared among mice in each group, and "stochastic gene profiles" (SGPs), i.e., unique union genes from one individual mouse to another. The CGPs of the experimental group and the SGPs of each individual mouse were separately characterized by individual assay. Concerning the CGPs, reciprocal strain differences between C3H/He and C57BL/6 mice in expression gene profiles, both plausible for leukemogenesis, were identified; namely, dominant downmodulations of *Sltm* and *Cryl1*, related to suppression of apoptosis and genomic instability in C3H/He mice, respectively, and dominant downmodulations of *Atrx/rad54* and *Kdm2a*, related to a decrease in DNA repair and genomic instability, respectively, in C57BL/6 mice. These findings imply that these reciprocal gene expression differences induced by benzene exposure may lead each strain to undergo different hematopoietic neoplastic pathways. In contrast, each individual mouse often shows a unique SGP. SGPs often include transcription factors, which regulate reciprocal signaling pathways including further SGPs. Among them, apoptosis-related genes expressed in C57BL/6 mice and those in C3H/He mice were attributable to different combinations of SGPs. Such stochastic case-by-case gene expression may be in good agreement with the individual and strain differences observed following benzene exposure. Because gene chip microarray techniques can elucidate stochastic changes in gene expression profiles, possible stochastic toxicology and its future role are discussed.

1. Introduction

Benzene-induced hematotoxic signaling is via aryl hydrocarbon-receptors (AhRs) [1] and specifically exhibited in hematopoietic progenitor cells [2,3]. The three major questions regarding benzene-induced hematopoietic neoplasms (HPNs)

Abbreviations: AML, acute myeloid leukemia; AhR, aryl hydrocarbon-receptor; HPNs, hematopoietic neoplasms.

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addressed are as follows: first, why is the incidence of HPNs equivocal in the case of low-dose benzene exposure despite the significant genotoxicity of benzene even at low doses [4]; second, why is there a plateau-like ceiling in the increase in the incidence of HPNs following high-dose exposure despite a low acute toxicity [5]? Third, why are acute myeloid leukemias (AMLs) *not* commonly observed in mice following benzene exposure even though they are frequently observed in humans after occupational benzene exposure [6–8]?

Since C3H/He mice are known to be AML-prone and to produce AMLs following radiation exposure [9,10], and C57BL/6 mice are known to be lymphoma-prone [11–13], these two strains were used for comparison. Owing the possible higher incidence of leukemias observed in *Trp*53-deficient mice [11,14,15], these mice of both C57BL/6 and C3H/He strains, were exposed to benzene (6 h/day, 5 days/week, for 26 weeks) and their leukemogenicities throughout their lifetimes were compared to determine whether *Trp*53-deficient C3H/He AML-prone mice, answer the above questions [11]. The followings are the results. For the first query, the apparently linear induction of HPNs following low-dose benzene exposure in *Trp*53-deficient mice was considered to be based on the DNA repair potential at low-dose level in wild-type mice. For the second query, the limited increase in the incidence of HPNs following high-dose benzene exposure was found to be due to excessive apoptosis in wild-type mice. For the last query, development of AML was observed in 38% of *Trp*53-deficient C3H/He mice [11].

Global gene expression analysis performed 28 days after a 2-week-intermittent benzene exposure at a dose of 150 mg/kg b.w./day for elucidation of the hemopoietic stem cell-specific, AhR-related low-dose adverse effect revealed reciprocal strain differences in relation to plausible hematopoietic leukemia/lymphomagenesis between C3H/He and C57BL/6 mice. We observed dominant downregulations of *Sltm* (scaffold attachment factor [SAF]-like transcription modulator) and *Cryl1* (crystallin, lambda 1), known to induce the suppression of apoptosis [16] and genomic instability, respectively [17,18] in C3H/He mice, and dominant downmodulations of *Atrx* (alpha thalassemia/mental retardation syndrome X-linked homolog)/*rad54* and *Kdm2a* (dimethyl Lys36 histone H3 [H3K36me2] histone demethylase), known to induce the suppression of DNA repair [19] and increase genomic instability [20], respectively, in C57BL/6 mice, implying that these reciprocal gene expression changes induced by benzene may lead to development of HPNs in both strains via different plausible neoplastic pathways toward the hematopoietic neoplasms.

2. Exposure doses and hematopoietic neoplasms

The hematotoxic signaling induced by benzene exposure is via AhRs [1]; thus, toxicity is specifically induced in hematopoietic progenitor cells of the bone marrow because they only specifically express AhRs during the steady-state [21–24]. Intermittent exposure of benzene at doses below 300 ppm by inhalation, 6 h/day, 5 days/week, for 26 weeks, was carried out so as not to induce aplastic anemia. Benzene exposure doses of 33, 100 and 300 ppm were therefore used along with sham exposure for comparison. Using the intermittent inhalation protocol with 300 ppm exposure dose, the incidence of leukemias induced was the maximum, which results in 30–50% of HPNs in general, depending on the mouse strain used [11,12,25–27]. Exposure concentrations of over 300 ppm tend to result in aplastic anemia and death in mice [25].

As results, first, the incidences of nonthymic lymphoma and AMLs in C3H/He mice showed apparently linear responses at the lower exposure dose in *Trp*53-deficient mice (Fig. 1A); second, the incidences of thymic lymphoma in C57BL/6 and nonthymic lymphoma in C3H/He mice increased without a plateau-like ceiling (Fig. 1B); thus, the former threshold-like equivocal induction of HPNs following low-dose benzene exposure is assumed to be based on the DNA repair potential in wild-type mice, and the latter limited increase in the incidence of HPNs following high-dose benzene exposure is due to excessive apoptosis owing to *Trp*53 in wild-type mice [11].

3. Incidence of myeloid leukemia in C3H/He mice

Concerning the incidence of AMLs, following exposure to 300 ppm benzene, 9% of the wild-type C3H/He AML-prone mice developed AMLs, whereas 38% of the *Trp*53-deficient mice developed AMLs (Fig. 2). Because AMLs were also observed in including *Trp*53-deficient C57BL/6, the lymphoma-prone mice, the induction of AMLs by benzene exposure was considered to be plausible not only in humans but also in mice with some strain differences. Further detailed incidences and histopathological findings observed in the study are described elsewhere [11].

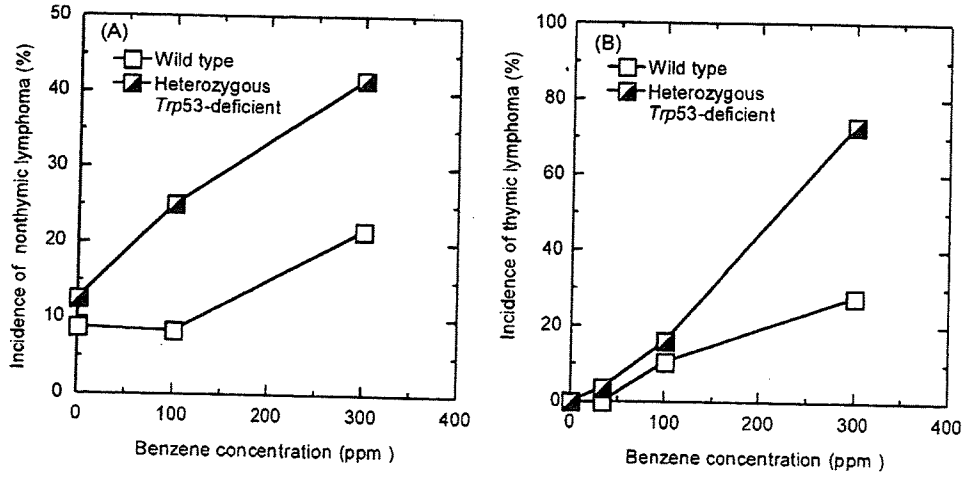

Fig. 1. Incidence of HPNs (percent) (ordinate) vs. dose of benzene exposure (abscissa): nonthymic lymphoma in C3H/He mice (A) and thymic lymphoma in C57BL/6 mice (B). Open square symbols represent wild-type mice, whereas half-closed square symbols represent heterozygous *Trp*53-deficient mice. Number of mice per group (%) of each data point is 2/23 (8.7), 2/24 (8.3), and 7/23 (30.4) for wild-type mice, and 5/24 (12.5%), 6/24 (25.0), and 10/24 (41.7) for heterozygous *Trp*-53-deficient mice for benzene exposure doses of 0, 100 and 300 ppm, respectively. The data are obtained from reference originally from [11].

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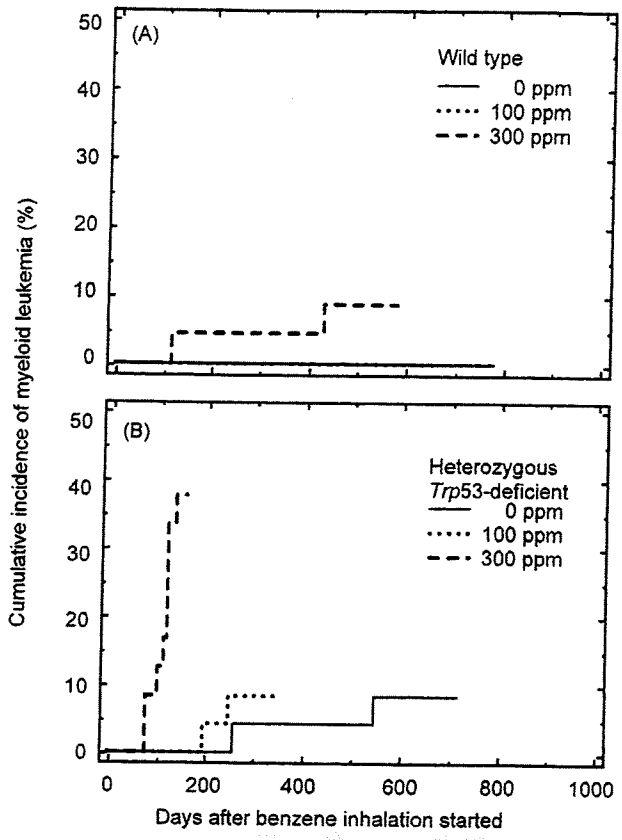

Fig. 2. Cumulative incidences of AMLs during the lifetime of C3H/He mice; wild-type mice (A) and heterozygous *Trp*53-deficient mice (B). Bold dotted lines indicate 300 ppm exposure dose, regular dotted lines indicate 100 ppm exposure dose, and solid lines indicate sham exposure controls. Statistical significance determined by log rank test: (A) no significant difference between groups; (B) 0 vs. 300 ppm, $p = 1.5 \times 10^{-4}$; 100 vs. 300 ppm, $p = 1.8 \times 10^{-4}$. Number of mice per group (%) at each data point is 0/23 (0), 0/24 (0), and 2/23 (8.7) for wild-type mice, and 2/24 (8.3%), and 2/24 (8.3), and 9/24 (37.5) for heterozygous *Trp53*-deficient mice for benzene exposure doses of 0, 100, and 300 ppm, respectively. The figures are taken from reference from [11].

4. Strain differences, reciprocal increase in genomic instability, and commonality-stochasticity relationship: microarray study

The dose of 150 mg/kg b.w./day for benzene exposure by gavage is the leukemogenic dose equivalent to the above-mentioned inhalation exposure dose, which induce similar incidences of HPNs through a lifetime [28,29]. Microarray analysis was performed after 2 weeks of intermittent gavage exposure to the equivalent dose of benzene. The 45,101 probe sets obtained were applied to an Affymetrix GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). We observed two conceptually different gene expression profiles; "common gene expression profiles" (CGPs) and "stochastic gene expression profiles" (SGPs).

Benzene exposure-specific gene expression intensities analyzed by two-way analysis of variance (ANOVA) with a p-value of less than 0.05 were considered statistically significant and a total of 258 probe sets were obtained (CGPs). Among them, five categories were identified by dendrogram analysis without any supervising information, consisting of four different reciprocal components, i.e., two strains with or without benzene exposure (data not shown). Category #1 consists of genes with upregulation during the steady-state and downregulation after benzene exposure predominantly in the C3H/He strain; Category #2 consists of genes with upregulation during the steady-state and downregulation after benzene exposure predominantly in the C57BL/6 strain; Category #4 consists of genes with upregulation during the steady-state as well as after benzene exposure predominantly in the C3H/He strain; and Category #5 consists of genes with upregulation during the steady-state as well as after benzene exposure predominantly in the C57BL/6 strain. Category #3 consists of genes with downregulation, but shows no expression difference between both strains, either during the steady-state or after benzene exposure. The characteristics of each category and sample representative common genes are shown in Table 1. Namely, Category #1 consists of *Cryl1* and *Sltm*; the former is known to be related to genomic instability [17,18], whereas the latter is known to be related to suppression of apoptosis [16]; thus, this category suggests plausible benzene-induced gene expression for C3H/He-dominant induction of hematopoietic disorders. Other C3H/He-dominated changes in Category #4 involve *WSTF/Baz1b* (Williams–Beuren syn-

Table 1
Sample representative common genes in each category.

Affymetrix system name	Common name	Genbank ID	Description
Category #1: steady-state upregulation (B6 < C3) and Bz-induced downregulation (B6 < C3)			
1447112_s_at	Cryl1	C85932	Crystallin, lambda 1
1424452_at	Sltm	BC019992	SAFB-like, transcription modulator
Category #2: steady-state upregulation (C3 < B6) and Bz-induced downregulation (C3 < B6)			
1435329_at	Kdm2a/Fbxl11	BE690994	Lysine (K)-specific demethylase 2A/F-box and leucine-rich repeat protein 11
1420947_at	Atrx/rad 54	BB825830	Alpha thalassemia/mental retardation syndrome X-linked homolog (human)
1423521_at	Lmnb1	AA270173	Lamin B1
1429658_a_at	Smc2	BI684556	Structural maintenance of chromosome 2
1450051_at	Atrx/rad 54	BB825830	Alpha thalassemia/mental retardation syndrome X-linked homolog (human)
1420946_at	Atrx/rad 54	BB825830	Alpha thalassemia/mental retardation syndrome X-linked homolog (human)
1449292_at	Rb1cc1	BE570980	RB1-inducible coiled-coil 1
1434045_at	Cdkn1b ($p27^{kip1}$)	BB354528	Cyclin-dependent kinase inhibitor 1B (P27)
Category #3: steady-state expression (C3 ≈ B6) and Bz-induced downregulation (C3 ≈ B6)			
Category #4: steady-state downregulation (B6 < C3) and Bz-induced upregulation (B6 < C3)			
1420975_at	Baz1b/WSTF	BB253608	Bromodomain adjacent to zinc finger domain, 1B/Williams syndrome transcription factor (WSTF)
1424875_at	Spg20	BB040507	Spastic paraplegia 20, spartin (Troyer syndrome) homolog (human)
1420849_at	Crnkl1	AV143435	Crn, crooked neck-like 1 (Drosophila)
Category #5: steady-state downregulation (C3 < B6) and Bz-induced upregulation (C3 < B6)			
1435054_at	Eme1	BG064903	Essential meiotic endonuclease 1 homolog 1 (*S. pombe*)

C3: C3H/He, B6: C57BL/6, Bz: benzene.

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drome transcription factor, also known as *BAZ1B*; a component of the WICH complex [WSTF-ISWI ATP-dependent chromatin-remodeling complex]), *Crnkl1* (Crn, crooked neck-like 1), and *Spg20* (spastic paraplegia 20, spartin [Troyer syndrome] homolog), the functions of which are related to enhancement of double-strand break repair [30], the cell cycle suppression [31], and upregulation of a potential suppressive factor [32], respectively, suggesting C3H/He-dominant reciprocal genomic stabilization against the above-mentioned function in Category #1.

C57BL/6-dominated CGPs for genomic stabilization are identified solely on the basis of the upregulation of *Eme1* (essential meiotic endonuclease 1 homolog 1, a component of the Mus81-Eme1 structure-specific endonuclease) in Category #5 and downregulation of *Lmnb1* (lamin B1) in Category #2, which repair DNA damages [33] associated with cell cycle suppression [34] after benzene exposure. The remaining genes identified in Category #2 are plausible genes that respond to possible neoplastic changes; such as downregulation of $p27^{kip1}$, which results in release from cell cycle suppression [35–37], downmodulation of *Kdm2a*, which increases genomic instability [20], downregulation of *Atrx/Rad54*, which suppresses DNA repair [19], downregulation of *Smc2* (structural maintenance of chromosomes 2), which suppresses double-strand break repair [38], and suppression of *Rb1cc1* (RB1-inducible coiled-coil 1), which increases in genomic instability owing to the suppression of Rb1-inducible genomic stabilization [39]. These are common trends observed in gene expression profiles for each strain or, in some cases for both strains, which are plausible gene expression changes in mice with benzene-induced HPNs.

In contrast to the above CGPs in the two strains, or either strain, each individual mouse often shows unique SGPs, as shown in Fig. 3 ("*stochastic gene expression*"). Such SGPs often include transcription factors, which regulate reciprocal signaling pathways including further SGPs. Each mouse among the five mice in the group shows expression intensities of each gene different from those of the others. The two panels on the top of Fig. 3 showing the apoptosis-related genes of C57BL/6 (left) and C3H/He (right) mice reveal the different SGP combinations for each animal (numbers 01–05 in C57BL/6 mice, and 06–10 in C3H/He) mice; Huntingtin interacting protein 1 (*HIP-1*), known to be an apoptosis inducer [40,41], is expressed in three out of five C57BL/6 mice but is not overexpressed in any C3H/He mice. Investigation of such stochastic, case-by-case gene expression requires a prohibitive number of cases to clusterize each signal statistically; however, this may eventually support the observed strain differences after benzene exposure.

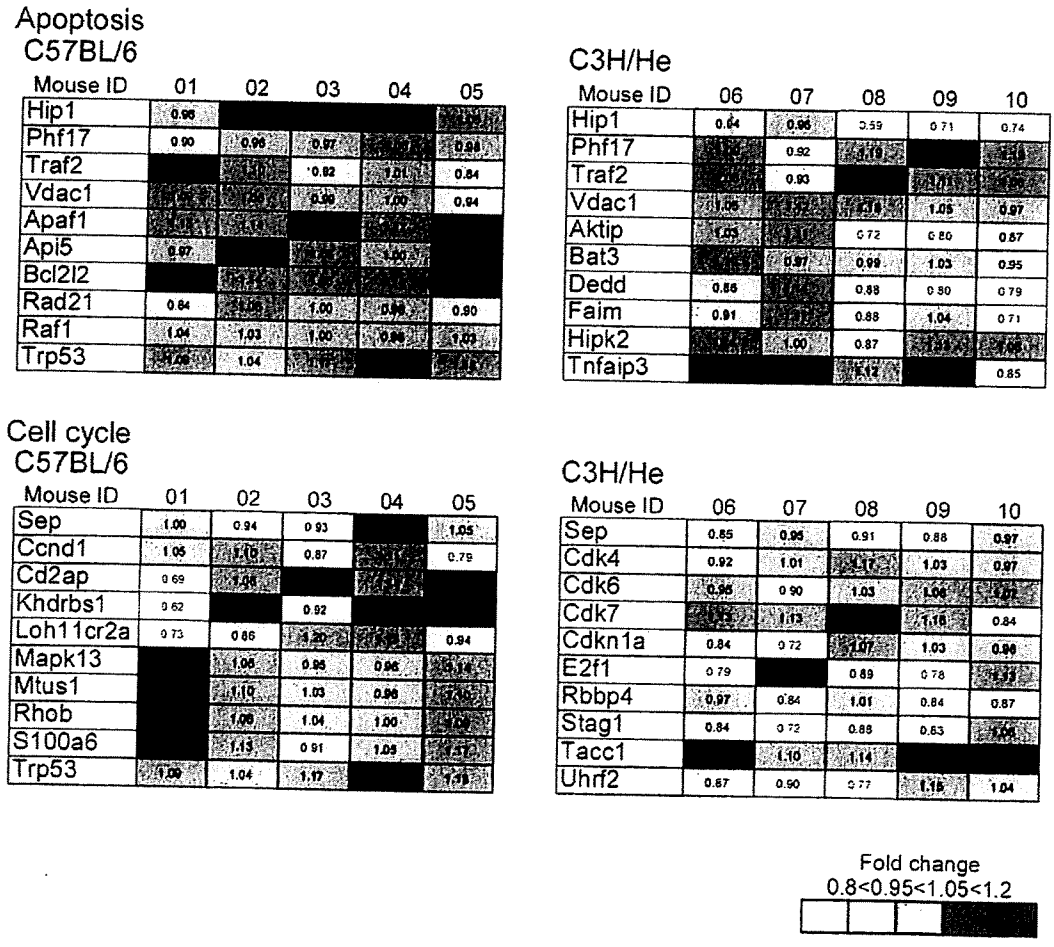

Fig. 3. Stochastic gene expression intensities in five mice of each strain: C57BL/6, 01–05 on the left; and C3H/He, 06–10 on the right. Ten representative genes for apoptosis, shown in the upper panels, and cell cycle-related genes, shown in the lower two panels are provided. Expression intensities are shown along the fold change scale in the bottom. Stochastic union genes were selected from each principal component analysis between a profile from one individual mouse and five other profiles from mice without benzene exposure, and total union genes with contribution scores above 0.9 or below −0.9 were collected; 1519 probe sets were obtained from C57BL/6 mice, and 1174 probe sets were obtained from C3H/He mice. Properties of selected union genes in each strain were analyzed by gene ontology (GO) and classified into categories, such as apoptosis-related or cell cycle-related.

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When one looks at the relationship between CGPs and SGPs in available gene expression signaling networks, possible bilateral relationships, i.e., centralizing signals from stochastic genes toward a commonly expressed gene, and diffusing signals from a commonly expressed gene to stochastic genes, are unsupervisedly identified. The CGPs are commonly shared among mice in each group, whereas the SGPs are unique union genes from one individual mouse to another. While the former profiles are definitive, and thus possibly diagnostic, the latter profiles are considered to be probabilistic and predictable if a number of cases can be clusterized statistically. Previous toxicological concept focused on the former definitive profiles simply owing to a lack of methodology for the latter. Because gene chip microarray techniques can now elucidate the above-mentioned stochastic changes, the newly developing field of stochastic toxicology may open up a world of new xenobiotic responses, which were ignored in previous toxicological studies as data of random dispersion [42,43].

Conflicts of interest statement

There are no conflicts of interest.

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

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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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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 ± 1 °C and 55 ± 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 pre-

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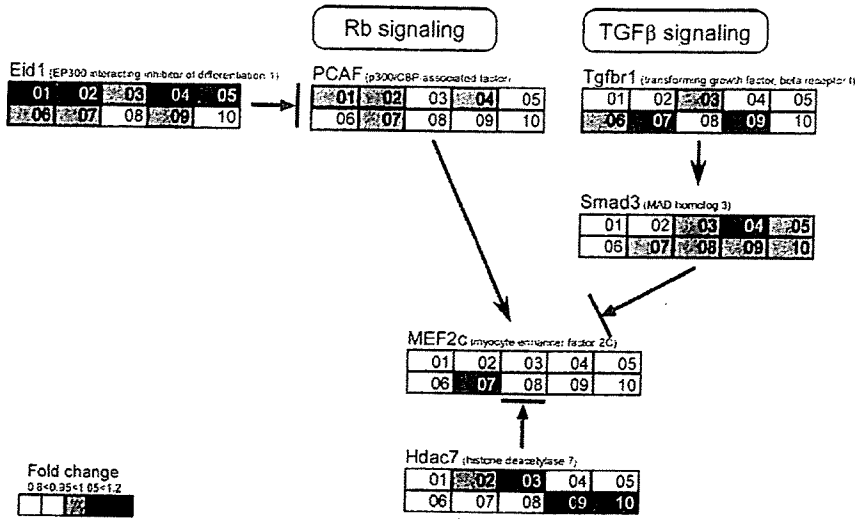

Fig. 1. Differential toxicities: hematopoietic progenitor cells of blood in the bone marrow (left panel) and mature blood cells in the liver (right panel). Both toxicities are transmitted by aryl hydrocarbon receptors. The former is based on cell-cycle arrest-related hematotoxicities and the latter is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

viously 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* 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

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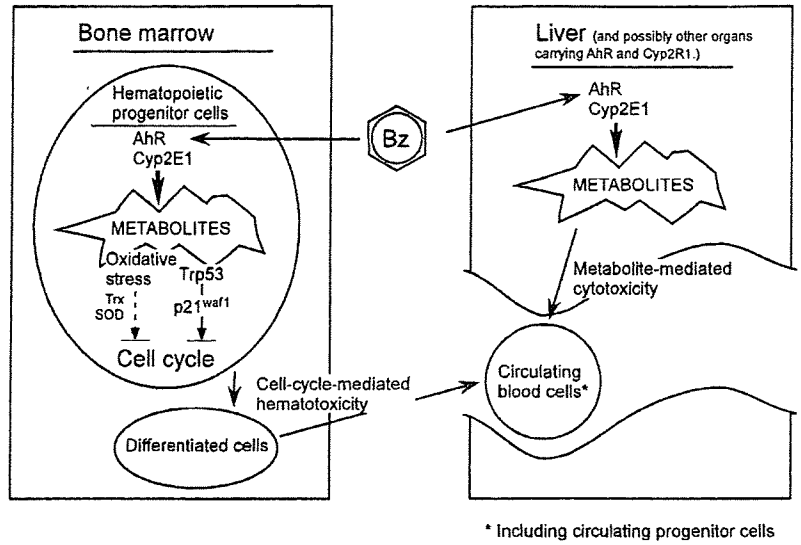

Fig. 2. Gene expressions in mice are designated with expression intensities with light to dark color in boxes separately from C57BL/6 mouse #1 (01), through #5 (05) in the upper row and from C3H/He mouse #6 (06) through #10 (10) in the lower row. *MEF2c*, a promoter of proliferation of pluripotent hematopoietic 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.

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 timecoarses 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

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from the LKS fraction, of which cell cycle impairment 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.

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Benzene-Induced Hematopoietic Neoplasms Including Myeloid Leukemia in *Trp*53-Deficient C57BL/6 and C3H/He Mice

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This research focused on three major questions regarding benzene-induced hematopoietic neoplasms (HPNs). First, why are HPNs induced equivocally and at only threshold level with low-dose benzene exposure despite the significant genotoxicity of benzene even at low doses both in experiments and in epidemiology? Second, why is there no linear increase in incidence at high-dose exposure despite a lower acute toxicity (LD_{50} > 1000 mg/kg body weight; WHO, 2003, *Benzene in drinking-water. Background document for development of WHO Guidelines for Drinking-Water Quality*)? Third, why are particular acute myeloid leukemias (AMLs) not commonly observed in mice, although AMLs are frequently observed in human cases of occupational exposure to benzene? In this study, we hypothesized that the threshold-like equivocal induction of HPNs at low-dose benzene exposure is based on DNA repair potential in wild-type mice and that the limited increase in HPNs at a high-dose exposure is due to excessive apoptosis in wild-type mice. To determine whether *Trp*53 deficiency satisfies the above hypotheses by eliminating or reducing DNA repair and by allowing cells to escape apoptosis, we evaluated the incidence of benzene-induced HPNs in *Trp*53-deficient C57BL/6 mice with specific regard to AMLs. We also used C3H/He mice, AML prone, with *Trp*53 deficiency to explore whether a higher incidence of AMLs on benzene exposure might explain the above human-murine differences. As a result, heterozygous *Trp*53-deficient mice of both strains showed a nonthreshold response of the incidence of HPNs at the lower dose, whereas both strains showed an increasing HPN incidence up to 100% with increasing benzene exposure dose, including AMLs, that developed 38% of heterozygous *Trp*53-deficient C3H/He mice compared to only 9% of wild-type mice exposed to the high dose. The detection of AMLs in heterozygous *Trp*53-deficient mice, even in the C57BL/6 strain, implies that benzene may be a potent inducer of AMLs also in mice with some strain differences.

Key Words: **benzene; acute myeloid leukemia; hematopoietic neoplasms; C57BL/6; C3H/He; *Trp*53-deficient mice.**

The association between chronic benzene exposure and its effect of hematopoietic impairment was first observed in 1897 in tire workers by Santesson (1897). As additional cases were accumulated (Delore and Borgomano, 1928; Le Noir and Claude, 1897; Selling, 1910; Cabot, 1927; Smith, 1928), researchers found that benzene exposure induced not only bone marrow (BM) failure/aplastic anemia but also hematopoietic neoplasms (HPNs) including leukemias (Aksoy *et al.*, 1974; Penati and Vigliani, 1938). As reported in the literature, there is a narrow benzene exposure range for HPNs including leukemias and that for reversible or irreversible marrow aplasia both in humans and in experimental animals. The association between the benzene exposure and the cause of HPNs remained unclear until 1980, when Snyder *et al.* (1980) observed the first HPNs in mice induced by lifetime benzene exposure at 300 ppm, 6 h/day, and 5 days/week. Subsequently, Cronkite *et al.* (1982, 1984) confirmed the induction of HPNs through an exposure protocol that referenced the number of hematopoietic progenitor cells noted during the course of treatment.

The groundbreaking intermittent exposure protocols, developed by Cronkite *et al.* (1982, 1984), was originally intended not to exhaust the target cells but to maintain hematopoietic stem cells capable of transforming into HPNs. Indeed, a very high dose of benzene exposure in Swiss mice administered by gavage (500 mg/kg body weight for 4–5 days/week for 78 weeks) failed to induce any HPNs (Maltoni *et al.*, 1989), whereas exposure at lower doses of benzene for even 2 years by gavage using a protocol similar to that developed by the groups of Snyder and Cronkite (0, 25, 50, and 100 mg/kg body weight, 5 days/week) was found to induce HPNs at incidences of 8, 21, 20, and 31%, respectively (Huff *et al.*, 1989; NTP, 1986).

Subsequent studies further disclosed that benzene-induced hematotoxicity is mediated by aryl hydrocarbon receptors (Yoon *et al.*, 2002). The Snyder-Cronkite's protocol of

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intermittent benzene inhalation was found to induce oscillatory proliferation of BM cells to counter any additional epigenetic hematopoietic neoplastic impacts (Yoon *et al.*, 2001). DNA repair systems would naturally be affected by such epigenetic neoplastic impacts during intermittent oscillatory changes, and the weak oxidative stress induced by benzene metabolites has also been found to influence neoplastic transformation (Li *et al.*, 2006; Snyder, 2007).

There remain some data gaps among the experimental animal studies of benzene-induced leukemias in this area. First, the incidence of HPNs after low-level benzene exposure in wild-type mice is threshold like and equivocal, despite the significant genotoxicity of benzene even at doses lower than 1 ppm and the related decrease in the number of hematopoietic progenitor cells (Lan *et al.*, 2004). Second, there is a nonlinear-plateaued increase in the incidence of HPNs despite the lower toxicity of benzene (large LD_{50} value of 1000–10,000 mg/kg body weight; WHO, 2003). Third, there is a lack of acute myeloid leukemias (AMLs) in most of the experimental studies in mice, despite the high frequency of AMLs observed in human cases of occupational exposure to benzene.

Accordingly, reevaluation is required in order to resolve these data gaps. The equivocal response of the induction of HPNs at low doses is hypothesized on the basis of DNA repair mechanisms in wild-type mice, while a limited increase in the incidence of HPNs at high doses is hypothesized for a highly apoptosis-sensitive subfraction in the BM. In exploring this hypothesis, *Trp*53 deficiency may prove useful since this deficiency provides a cellular mechanism for the failure of DNA repair and for escape from apoptosis (French *et al.*, 2001; Hirabayashi *et al.*, 2003; MacDonald *et al.*, 2004; Storer *et al.*, 2001). *Trp*53-deficient C57BL/6 mice were used to evaluate the incidence of benzene-induced HPNs, specifically, in AMLs where there is a lack of DNA repair. Any potential increase in incidence of HPNs due to known *Trp* deficiency mechanisms may be interpreted in relation to murine AMLs. We can then compare the development of AML in these *Trp*53-deficient C57BL/6 mice to that seen in a C3H/He (AML prone) strain. This will make it feasible to identify any differences among strains regarding potentially excessive induction of leukemia associated with *Trp*53 deficiency.

*Trp*53-deficient mice show increased genomic instability and deficient repair mechanism because of the absence of cell cycle arrest induced by *Trp*53 after genotoxic damage. These mice, thus, provide a useful tool for examining an exaggerated neoplastic transformation after DNA damage induced by genotoxic chemicals. A marked increase in the incidence of chemical-induced cancers is potentially attributable to a genotoxic mechanism (Harvey *et al.*, 1993; Hirabayashi *et al.*, 2003; MacDonald *et al.*, 2004; Kemp *et al.*, 1994; Yoshida *et al.*, 2007). This method has also been recommended as a sensitive experimental tool for carcinogenicity bioassay of directly genotoxic carcinogens, for ionizing radiation as well as for chemicals (French *et al.*, 2001; Hirabayashi *et al.*, 2003; MacDonald *et al.*, 2004; Storer *et al.*, 2001). However, homozygous *Trp*53-deficient mice are difficult to utilize because of the high frequency of spontaneous thymic lymphomagenesis (Hirabayashi *et al.*, 2003; MacDonald *et al.*, 2004) due to the lack of physiological apoptosis in the double-negative immature T-cell subpopulation during the developmental stage (Haines *et al.*, 2006).

Because the C3H/He strain exhibits a relatively high incidence of AML (Seki *et al.*, 1991; Yoshida *et al.*, 1996), the use of *Trp*53-deficient mice from both the C57BL/6 strain and the C3H/He strain may elucidate potential relationships and differences between benzene exposure and the development of AMLs in these two strains.

Owing to the high neoplastic sensitivity and myeloid leukemogenicity of the heterozygous *Trp*53-deficient C3H/He mice use in this study, exposure to benzene induced strain-dependent HPNs, including AMLs, in a nearly benzene dose–dependent manner, suggesting that our findings on the heterozygous *Trp*53-deficient mouse may provide a useful experimental model for studying benzene-induced hematotoxicity.

MATERIALS AND METHODS

Benzene. Benzene (CAS. no. 71-43-2, MW 78.11), widely utilized as a solvent for a various organic chemicals and present in gasoline and tobacco cigarettes, was obtained from Wako Fine Chemicals (Tokyo, Japan).

Animals. The targeting vector for *Trp*53, a recombinant with a 2.8-kb vector containing a neomycin-resistant gene immediately before the transcriptional start site, was inserted into TT2 embryonic stem cells (heterozygous for C57BL/6 and CBA; Yagi *et al.*, 1993) to established homologous recombinant clones (Tsukada *et al.*, 1993). By generating aggregation chimeras with this recombinant clones, chimeric mice and then *Trp*53-knockout mice were established in 1987 after confirmation of the germinal transmission in *Trp*53-deficient (C57BL/6 × CBA) F1 mice (Tsukada *et al.*, 1993). General information on these recombinant mice is also found elsewhere (*Trp*53^{tm1Sia} MGI: 1926340, Mouse Genome Informatics, 2009). The original *Trp*53-deficient (C57BL/6 × CBA) F1 mice backcrossed with C57BL/6 were transferred to the animal facility of the National Institute of Health Sciences (NIHS), Japan, in the second generation. Since then, the backcrossing with C57BL/6CrSlc was carried out for over 20 generations in 1997, followed by backcrossing with C3H/HeMsNrs in 2002. Both *Trp*53-deficient strains, C57BL/6 and C3H/He, were maintained by repeated backcrossing for each strain continuously.

This study used wild type, and homozygous and heterozygous *Trp*53-deficient male C57BL/6 and C3H/He mice were used. The heterozygous and homozygous *Trp*53-deficient mice and wild-type mice were generated by mating between heterozygous *Trp*53-deficient mice at the animal facility of NIHS, Japan. Neonates were genotyped using the primer for the targeted DNA sequence, including a partial *neo* gene at the 5′ end partial exon 4, by PCR analysis using tissues obtained from the tail (Hirabayashi *et al.*, 2002; Tsukada *et al.*, 1993; Yoshida *et al.*, 2002).

Cohort studies using 8-week-old mice were conducted using 10 mice for each genotype each time. Only male mice were studied in each strain owing to the similar incidences of HPN induction in both genders and to a limited number of rooms in the animal facility with gas chromatographs for the accurate monitoring of benzene exposure concentration. C57BL/6 mice (all genotypes) totaled 76 wild-type mice, 102 heterozygous *Trp*53-deficient mice, and 86 homozygous *Trp*53-deficient mice. All the animals were randomly selected on the basis of body weight and grouped by benzene dosage (300, 100,

TABLE 1
Incidences of Hematopoietic and Nonhematopoietic Diseases (histopathological types, C57BL/6 mice)

Genotype	Wild type				Heterozygous *Trp53* deficiency				Homozygous *Trp53* deficiency			
Benzene dose	0	33	100	300	0	33	100	300	0	33	100	300
No. of mice/group	20	19	19	18	24	27	25	26	21	19	23	23
HPNs (%)	2 (10.0)	4 (21.0)	3 (15.8)	10 (55.6)*	937.5%	11(40.7)	9 (36.0)	23 (88.5)*	19 (90.5)	18 (94.7)	22 (95.7)	17 (73.9)
Thymic lymphoma (%)	0 (0.0)	0 (0.0)	2 (10.5)	5 (27.8)*	0 (0.0)	1 (3.7)	4 (16.0)	19 (73.1)*	11 (52.4)	11 (57.9)	13 (56.5)	12 (52.2)
Nonthymic lymphoma (%)	2 (10.0)	4 (21.0)	1 (5.2)	5 (27.8)	9 (37.5)[a]	10 (37.0)	5 (20.0)	2 (7.7)*	8 (38.1)	7 (36.8)	8 (34.8)	5 (21.7)
Myeloid leukemia (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (7.7)	0 (0.0)	0 (0.0)	1 (4.3)	0 (0.0)
Other hematopoietic disorders (%)	2 (10.0)	1 (5.2)	1 (5.2)	0 (0.0)	5 (20.8)	4 (14.8)	1 (4.0)	1 (3.8)	2 (9.5)	0 (0.0)	0 (0.0)	0 (0.0)
Malignant fibrous histiocytoma (%)	0 (0.0)	1 (5.2)	1 (5.2)	0 (0.0)	5 (20.8)	3 (11.1)	0* (0.0)	0 (0.0)*	2 (9.5)	0 (0.0)	0 (0.0)	0 (0.0)
Myeloproliferative disorders/ myelodysplastic syndrome (%)	2 (10.0)	4 (21.1)[b]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Aplastic anemia/marrow failure (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.7)	1 (4.0)	1 (3.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Nonhematopoietic solid tumors (%)	3 (15.0)	3 (15.8)[b]	8 (42.1)	2 (11.1)	6 (25.0)[a]	12 (44.4)	8 (32.0)	2 (7.7)	0 (0.0)	1 (5.3)	1 (4.3)	6 (26.1)*
Non-neoplastic fatal diseases (%)	13 (65.0)	11 (57.9)[b]	7 (36.8)	6 (33.3)	5 (20.8)	0 (0.0)	7 (28.0)	0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a]Mouse that has two diseases.
[b]Other hematopoietic disorders and nonhematopoietic solid tumors or non-neoplastic fatal diseases overlapped.
*p Values < 0.05 between each sham control and treated group by Fisher's exact test.

33, and 0 ppm [sham exposure control]). Table 1 shows final numbers for all mice after the start of benzene exposure.

Totals for C3H/He mice were 70 wild-type mice, 72 heterozygous *Trp53*-deficient mice, and 60 homozygous *Trp53*-deficient mice. After random selection based on body weight, the mice were divided into three groups by benzene dosage (300, 100, and 0 ppm [as sham exposure control]). Table 2 shows final numbers for all mice after the start of benzene exposure.

During the study, the mice were housed individually within stainless wire cages, placed in inhalation chambers, and were kept on a 12-h light-dark cycle. An autoclave-sterilized basal pellet diet (CRF-1, Oriental Yeast Co., Ltd, Tokyo, Japan) was provided *ad libitum*, except during the 6-h daily inhalation time, when food was withdrawn irrespective of benzene treatment. Ultraviolet-sterilized water was supplied automatically via a tube throughout the study.

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, a peer review panel established at NIHS, and approved by the Committee for Animal Care and Use (CACU) of the NIHS with the experimental code #473-2006. All animal studies were conducted using humane protocols approved by the CACU of the NIHS, Japan.

Benzene exposure. The mice were divided into the sham exposure control and benzene-exposed groups and housed in 1.3-m^3 horizontal lamina flow inhalation chambers with a flow rate of 650 l/min and 26 ventilation times/h (Sibata Scientific Technology Ltd., Tokyo, Japan) (Li *et al.*, 2006; Yoon *et al.*, 2001, 2002, 2003). The experimental groups were exposed to benzene at 300, 100, and 33 ppm for C57BL/6 mice and at 300 and 100 ppm for C3H/He mice, 6 h/day, 5 days/week for 26 weeks. The sham exposure control mice were maintained under the same conditions without benzene inhalation. After 26

TABLE 2
Incidences of Hematopoietic and Nonhematopoietic Diseases (histopathological types, C3H/He mice)

Genotype	Wild type			Heterozygous *Trp53* deficiency			Homozygous *Trp53* deficiency		
Benzene dose	0	100	300	0	100	300	0	100	300
No. of mice/group	23	24	23	24	24	24	18	20	22
HPNs (%)	2 (8.7)	6 (25.0)	7 (30.4)	6 (25.0)	20 (83.3)*	25 (104.2)*	15 (83.3)	14 (70.0)	20 (90.9)
Thymic lymphoma (%)	0 (0.0)	4 (16.7)	0 (0.0)	1 (4.2)	12 (50.0)*	6 (25.0)*,[a]	12 (66.7)	12 (60.0)[a]	15 (68.2)[a]
Nonthymic lymphoma (%)	2 (8.7)[a]	2 (8.3)	5 (21.7)[a]	3 (12.5)	6 (25.0)	10 (41.7)*,[a]	2 (11.1)	1 (5.0)	4 (18.2)
Myeloid leukemia (%)	0 (0.0)	0 (0.0)	2 (8.7)	2 (8.3)	2 (8.3)	9 (37.5)*	1 (5.6)	1 (5.0)	1 (4.6)[a]
Other hematopoietic disorders (%)	1 (4.4)	6 (25.0)	2 (8.7)	0 (0.0)	1 (4.2)	0 (0.0)	0 (0.0)	4 (20.0)	2 (9.1)
Malignant fibrous histiocytoma (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.2)	0 (0.0)	0 (0.0)	1 (5.0)	2 (9.1)
Myeloproliferative disorders/ myelodysplastic syndrome (%)	1 (4.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Aplastic anemia/marrow failure (%)	0 (0.0)	6 (25.0)*	2 (8.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (15.0)	0 (0.0)
Nonhematopoietic solid tumors (%)	11 (47.8)[a]	5 (20.8)*	8 (34.8)[a]	11 (45.8)	2 (8.3)*	0 (0.0)*	0 (0.0)	2 (10.0)[a]	1 (4.6)
Non-neoplastic fatal diseases (%)	10 (43.5)	7 (29.2)	7 (30.4)	7 (29.2)	1 (4.2)*	0 (0.0)*	3 (16.7)	1 (5.0)	0 (0.0)

*p Values < 0.05 between each sham control and treated group by Fisher's exact test.
[a]Mouse that has two diseases.

weeks, all the animals were observed throughout their lifetime under the same conditions without benzene inhalation.

Dose monitoring for benzene exposure. The benzene atmosphere was generated by heating liquid benzene to 16°C to form a vapor (Sibata Scientific Technology Ltd). A gas chromatograph (Shimadzu Co., Kyoto, Japan) was used to measure benzene concentration in the chambers at 30-min intervals during daily exposures (Shimadzu Co.) (Li *et al.*, 2006; Yoon *et al.*, 2001, 2002, 2003). The temperature and humidity in the chambers were automatically controlled at 24°C ± 1°C and 55 ± 10%, respectively.

PCR analysis for genotyping. To detect *Trp*53 wild-type and *Trp*53-deficient alleles, PCR analysis was performed using genomic DNA extracted from the tail of each mouse, and synthetic oligonucleotides were used as primers as described elsewhere (Tsukada *et al.*, 1993) and briefly here as follows. To detect the *Trp*53 wild-type allele, the common 5′ primer (5′-aattgacaagttatgcatcca-3′) and 3′ primer (5′-actcctcaacatcctggggcagcaacagat-3′) were used. To detect the *Trp*53-deficient allele, the common 5′ primer and *neo sequence* primer (5′-gaacctgcgtgcaatccatcttgttcaatg-3′) were used.

Lifetime observation. All mice were monitored at least twice daily throughout their lifetime. Those showing fatal symptoms, including advanced leukemias, such as anemia with pale extremities and palpable splenomegaly, were euthanized at the agonal period and then examined hematopathologically and histopathologically. Mice that died were examined for their gross anatomical features, after which all visceral organs were fixed in 10% neutral buffered formalin for histopathological examination.

Histopathological examination. All visceral organs, including the thymus, spleen, sternum, and femoral BM, were fixed in 10% neutral buffered formalin for 24 h. The sternum and femoral BM were decalcified in 7.5% formic acid for 72 h. After conventional processing for dehydration, paraffin-embedded sections were stained with hematoxylin and eosin and then examined histopathologically under a light microscope (Frith *et al.*, 2001; Hirabayashi *et al.*, 1992).

Loss of heterozygosity. During the course of benzene-induced leukemogenesis, the remaining wild-type allele of *Trp*53 remaining in heterozygous *Trp*53-deficient mice may be inactivated. The frequency of such loss of heterozygosity (LOH) was previously evaluated in mice with radiation-induced leukemias. LOH for the remaining *Trp*53 allele was not examined in each group because high level of consistency (91.7%) had been identified in the leukemogenicity assay previously conducted for this strain at our laboratory (Yoshida *et al.*, 2007).

Statistical analyses. Survival curves data were stored in a computer and processed for statistical analysis to obtain mean survival time and SE by the Kaplan-Meier method and to evaluate statistical significance by the log-rank test using SPSS 14.1 (SPSS, Inc., Chicago, IL). To determine the cumulative incidences of diseases, Fisher's exact test was applied using Microsoft Office Excel 2003 (Microsoft, Redmond, WA). Differences were considered significant at $p < 0.05$.

RESULTS

Survival Curves with Graded Exposure Doses of Benzene Inhalation

Experimental groups. Kaplan-Meier survival curves for wild-type mice in comparison to the two strains (C57BL/6 and C3H/He) of heterozygous and homozygous *Trp*53-deficient mice are shown in Figures 1A–1C and Figures 1D–1F, respectively. Figures 1A–C show data for C57BL/6 mice of different genotypes, classified into four groups on the basis of benzene exposure (33, 100, and 300 ppm, 6 h/day, 5 days/week, for 26 weeks, and 0 ppm as the sham exposure control). Figures 1D–1F show data from C3H/He mice of different genotypes classified into three groups on the basis of benzene exposure dose (100 and 300 ppm, 6 h/day, 5 days/week, for 26 weeks, and 0 ppm as the sham exposure control).

C57BL/6 strain. The mean survival time for C57BL/6, wild-type mice in the sham exposure control group, was 629 ± 40 days (mean ± SE) after the start of the experiment (Fig. 1A). The mean survival time for the wild-type mice in the 33- and 100-ppm exposure groups was 635 ± 40 and 550 ± 41 days, respectively, and the mean survival times for wild-type mice in the 300-ppm exposure group was 346 ± 30 days (mean ± SE). Survival time decreased proportionally with increasing benzene exposure except for the slight overlapping of survival curves for the 33-ppm and the sham exposure control groups.

Among heterozygous *Trp*53-deficient mice in the 300-ppm exposure group (Fig. 1B), the survival curve shows a rapid decrease in the number of surviving mice. Mean survival time in this group was 163 ± 9 days (mean ± SE) after the start of exposure, in comparison to 346 ± 30 days in the wild-type mice. Thus, the mean survival times for the heterozygous *Trp*53-deficient group and the wild-type group, both exposed to 300 ppm, were 347 and 283 days, respectively, shorter than the corresponding sham exposure control groups (510 ± 25 days for the heterozygous *Trp*53-deficient group and 629 ± 40 days for the wild-type group).

C3H/He strain. In the C3H/He wild-type sham exposure group, survival time was 590 ± 33 days (mean ± SE) after the start of exposure (Fig. 1D). Mean survival time in the wild-type 100-ppm exposure group was 495 ± 39 days and in the wild-type 300-ppm exposure group was 353 ± 35 days (mean ± SE in both cases). In contrast, within the heterozygous *Trp*53-deficient group exposed to 300-ppm benzene by inhalation, the first death occurred about 71 days after the start of exposure, and mean survival time ± SE was 117 ± 5 days (Fig. 1E).

Homozygous Trp53-deficient mice. All mice in both strains with homozygous *Trp*53 deficiencies died relatively soon (Figs. 1C and 1F), with mean survival times ranging 16–122 days, regardless of benzene exposure including 0 ppm. All survival curves, specifically in the four C57BL/6 groups, crossed or nearly crossed each other, except for longer survival in a small number of mice (less than 5%) in the 100-ppm exposure group. We attribute this to primarily thymic lymphomas that originate in double-negative CD4/CD8 cells lacking apoptosis, so our findings in homozygous *Trp*53-deficient mice have been omitted from further discussion. In the C3H/He mice, however, Kaplan-Meier comparison showed a statistically significant difference in survival curves between the 300-ppm exposure group and the sham exposure as determined by the log-rank test ($p = 0.002$, data not shown).

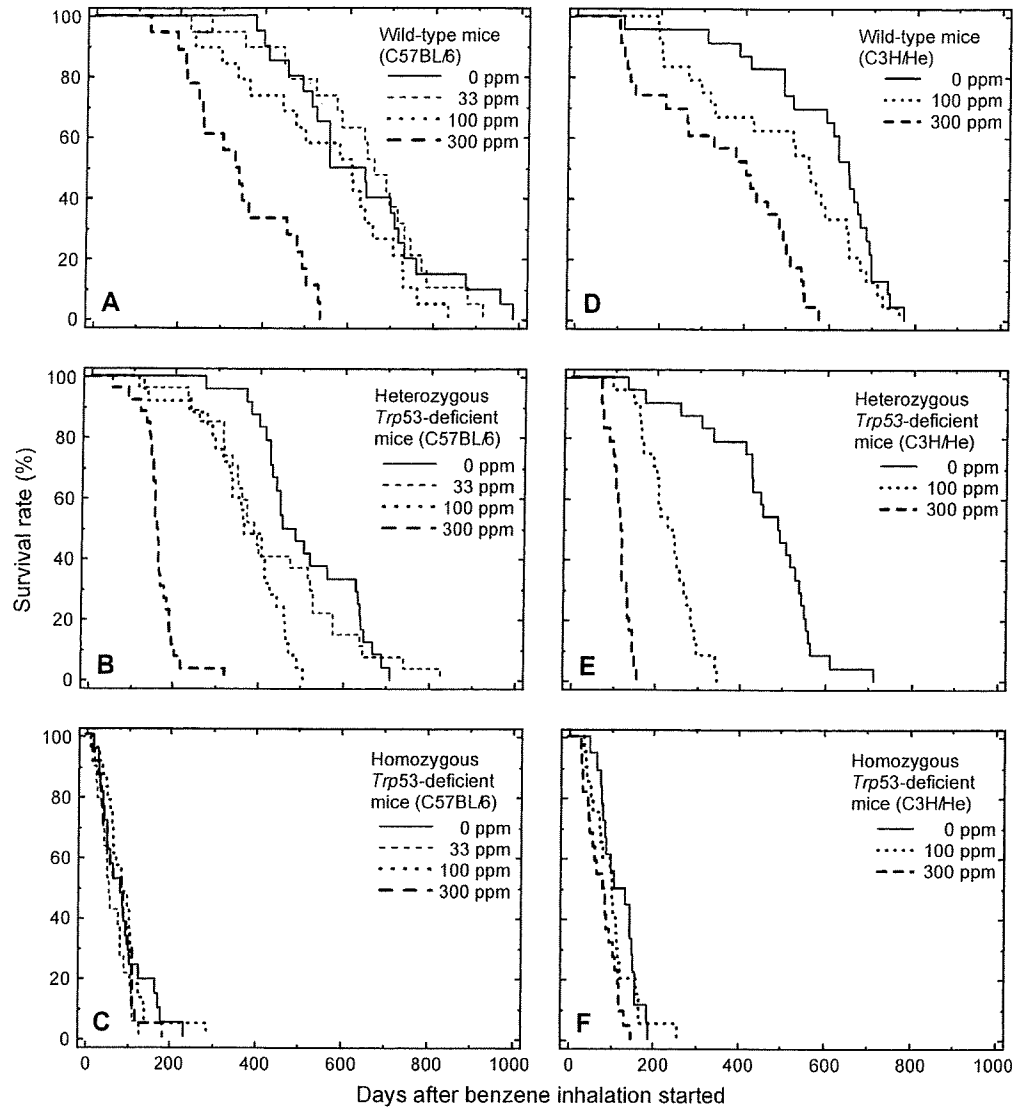

FIG. 1. Survival rates (%) are plotted on the vertical axis and survival time (days) after benzene inhalation on the horizontal axis, using (A)–(C) for C57BL/6 mice and (D)–(F) for C3H/He mice, with (A) and (D) for wild-type mice, (B) and (E) for heterozygous *Trp*53-deficient mice, and (C) and (F) for homozygous *Trp*53-deficient mice. Bold dotted lines for 300-ppm exposure group, regular dotted line for 100-ppm exposure group, fine dotted lines for 33-ppm exposure group, and solid line for sham exposure control. Statistical significance by log-rank test: (A) 0 versus 300 ppm, $p = 4.7 \times 10^{-06}$; 33 versus 300 ppm, $p = 8.3 \times 10^{-06}$; 100 versus 300 ppm, $p = 4.9 \times 10^{-04}$; (B) 0 versus 300 ppm, $p = 1.4 \times 10^{-10}$; 33 versus 300 ppm, $p = 4.8 \times 10^{-10}$; 100 versus 300 ppm, $p = 2.2 \times 10^{-08}$; 0 versus 100, $p = 1.9 \times 10^{-04}$; 33 versus 100 ppm, $p = 3.5 \times 10^{-02}$; (C) no significant difference between groups; (D) 0 versus 300 ppm, $p = 5.6 \times 10^{-06}$; 100 versus 300 ppm, $p = 1.0 \times 10^{-03}$; (E) 0 versus 300 ppm, $p = 5.8 \times 10^{-06}$; 100 versus 300 ppm, $p = 4.0 \times 10^{-09}$; 0 versus 100 ppm, $p = 1.1 \times 10^{-07}$; and (F) 0 versus 300 ppm, $p = 4.4 \times 10^{-03}$.

Cumulative Deaths due to HPNs

Benzene exposure in wild-type mice. The cumulative incidences of HPNs in each wild-type experimental group are shown in Figure 2A (C57BL/6) and Figure 2D (C3H/He). In C57BL/6 mice, the wild-type group exposed to 300 ppm showed a gradual increase in cumulative incidence of HPNs to 55.6% by day 532. In C3H/He mice, groups exposed to 100 and 300 ppm showed somewhat lower but similar increases in HPNs to 25.0% by 554 days and 30.4% by 431 days, respectively, as seen in Figure 2A (C57BL/6) and Figure 2D (C3H/He). With the exception of the 300-ppm exposure group of wild-type C57BL/6 mice, the incidence and onset of HPNs did not exceed 21.0% during lifetime observation (21.0% for the 33-ppm group and 15.8% for the 100-ppm groups). The maximum incidences of HPNs in the wild-type sham control group were 10.0% by 492 days in C57BL/6 mice and 8.7% by 742 days in C3H/He mice.

The first question in the present study concerned threshold-like equivocal incidence of HPNs at low-dose benzene exposure. In this regard, only the C57BL/6, 300-ppm exposure

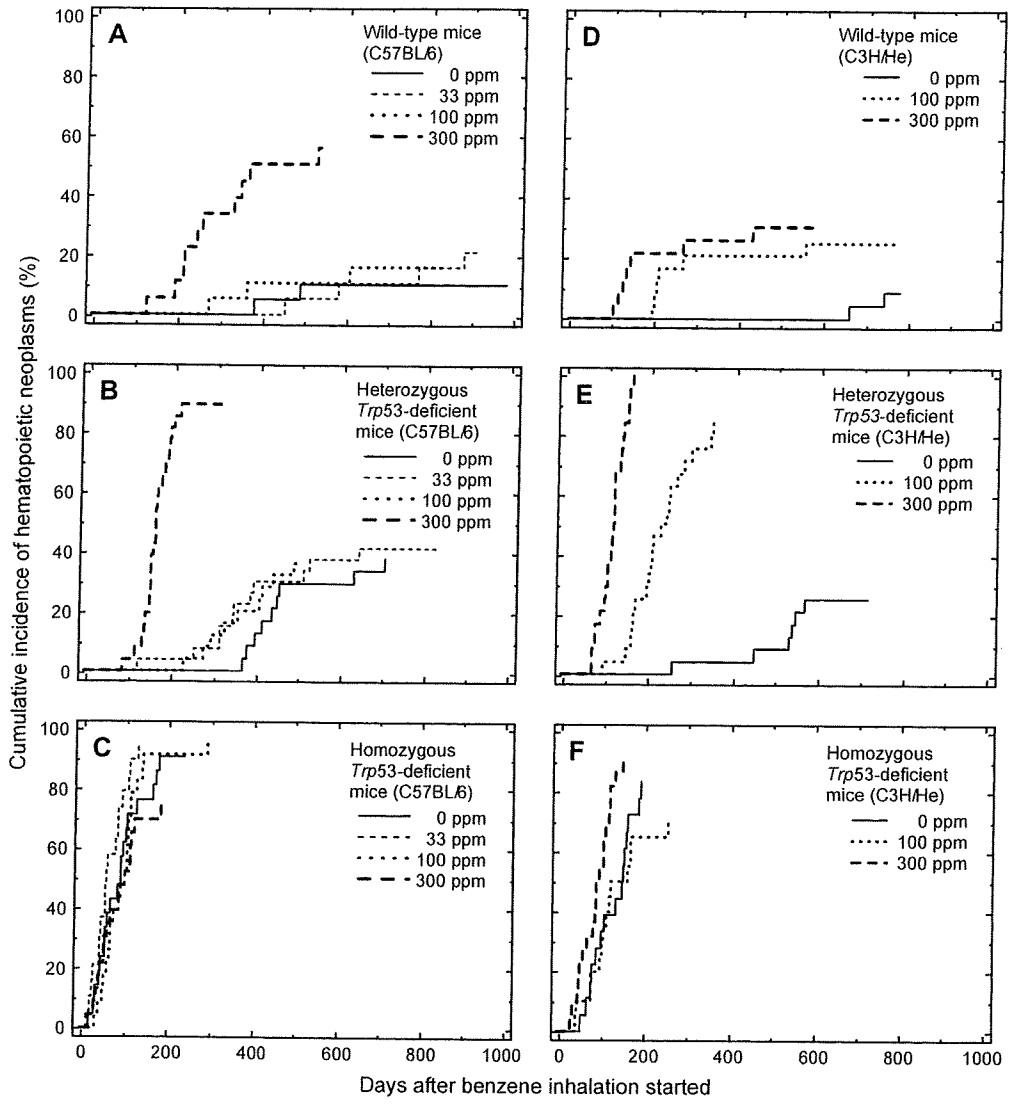

FIG. 2. The illustration shows the cumulative lifetime incidence of all hematopoietic malignancies (%) in C57BL/6 mice on the left (A–C) and in C3H/He mice on the right (D–F), using (A) and (D) for wild-type mice, (B) and (E) for heterozygous p53-deficient mice, and (C) and (F) for homozygous p53-deficient mice. Bold dotted line for 300-ppm exposure group, regular dotted line for 100-ppm exposure group, fine dotted line for 33-ppm exposure group, and solid line for sham exposure control. Statistical significance by log-rank test: (A) 0 versus 300 ppm, $p = 2.7 \times 10^{-04}$; 33 versus 300 ppm, $p = 4.5 \times 10^{-04}$; 100 versus 300 ppm, $p = 1.8 \times 10^{-03}$; (B) 0 versus 300 ppm, $p = 2.5 \times 10^{-10}$; 33 versus 300 ppm, $p = 8.6 \times 10^{-10}$; 100 versus 300 ppm, $p = 9.1 \times 10^{-10}$; (C) no significant difference between groups; (D) 0 versus 300 ppm, $p = 7.0 \times 10^{-03}$; (E) 0 versus 300 ppm, $p = 1.2 \times 10^{-11}$; 100 versus 300 ppm, $p = 2.8 \times 10^{-09}$; 0 versus 100 ppm, $p = 4.2 \times 10^{-08}$; and (F) 0 versus 300 ppm, $p = 3.1 \times 10^{-03}$; 100 versus 300 ppm, $p = 2.1 \times 10^{-02}$.

group showed a significant differences in cumulative HPN incidence in comparison to the other C57BL/6 groups. However, findings from both the C3H/He 300-ppm and the 100-ppm exposure groups differed significantly from the sham exposure controls. These results imply that HPNs occurred at a higher than threshold level in heterozygous *Trp*53-deficient mice in both strains since such incidence was greater than and clearly separated from the incidence in each sham exposure control groups.

Exposure in Trp53-deficient mice. A high frequency of HPNs was observed in both strains of the heterozygous *Trp*53-deficient benzene exposure groups as shown in Figure 2B (C57BL/6) and Figure 2E (C3H/He). In heterozygous *Trp*53-deficient C57BL/6 mice, a total HPN incidence of 88.5% (300 ppm) was observed from 88 to 219 days. This incidence was higher than in the sham exposure control (37.5%) and also higher than in the wild-type groups with or without benzene exposure (55.6 and 10.0%, respectively) and with earlier onset time (88 days) than in wild-type mice (130 days). The increase in incidence of HPNs between benzene exposure group and sham control was not greater in *Trp*53-deficient C57BL/6 mice than in the wild-type mice due to an increase in the late-appearing spontaneous HPNs in the *Trp*53-deficient mice, but the 50% die-off time (days) for HPNs between the former and