Table 1 – Reproductive toxicology in AhR-deficiencies.

Genotype of parents		No. of pups (sets[a])	Sex		Genotype		
Mother	Father		Male	Female	+/+	+/−	−/−
			(Genotype: $AhR^{+/+}$, $^{+/-}$, $^{-/-}$)		(Sex: male, female)		
AhR+/+	AhR+/−	154 (18)	71 (46, 25, –)	83 (50, 33, –)	96 (46, 50)	58 (25, 33)	– (–, –)
AhR+/−	AhR+/−	206 (27)	100 (36, 48, 16)	106 (32, 53, 21)	68 (36, 32)	101 (48, 53)	37 (16, 22)

[a] Sets for mating: each set consists of 2 females and 1 male.

hemizygous males, show a greater dysfunction in fertilization than wild-type male mice. For example, during reproduction in the current generation, crossbreeding between hemizygous AhR-knockout (KO) male mice and wild-type female mice produced 71 males and 83 females (Table 1). Among these, 96 mice were of the wild type and 58 mice were hemizygous AhR-KO. Although the number of puppies of wild-type mice and that of hemizygous AhR-KO mice were supposed to be equal in accordance with the Mendelian law, the number of puppies of hemizygous AhR-KO mice (58) was 60.4% lower than that of wild-type mice (96). Interestingly, this low incidence seemed to be largely based on the hemizygous AhR-KO males, because, among the 71 male puppies mentioned above, 25 were hemizygous AhR-KO mice, and 46 were of the wild type, which showed a statistically significant difference ($p < 0.028$). Among the 83 puppies mentioned above, 33 puppies were female hemizygous AhR-KO mice and 50 female puppies were of the wild type, which showed a statistically insignificant difference ($p > 0.064$). Furthermore, the mating study disclosed a more detailed underlying mechanistic background of reproductive dysfunction in male AhR-KO mice. When hemizygous AhR-KO males and their corresponding females were crossbred, the numbers of puppies observed were 100 males and 106 females (Table 1). Among them, the numbers of puppies were 37 homozygous AhR-KO mice, 101 hemizygous AhR-KO mice, and 68 wild-type mice. Because the average number of puppies of C57BL/6 mice at the animal colony was 6–7 mice per litter, the number of puppies observed in the present study was slightly larger than this average (132%), that is, 51.5 puppies, calculated from the total number of puppies (206) divided by 4 (25%) on the basis of the Mendelian law. These results imply that the incidence of homozygous AhR-KO mice was 45.6% lower than the incidence expected from the Mendelian law. Furthermore, this lower incidence is not supposed to be due to the death of AhR-KO puppies; but rather, AhR-KO puppies were supposed to have shifted to the wild type, because the cross-breeding was between hemizygous AhR-KO mice, in which sperms derived from the testicular glands of AhR-KO and wild-type may have competed during fertilization for fertilizing function. Indeed, when homozygous AhR-KO mice were crossbred, neither their litter size nor the ratio of the number of males to that of females was statistically significantly different from that of the wild type, because the competitive disadvantages observed in the hemizygous AhR-KO mice may have been negated by the same testicular dysfunctions in the homozygous AhR-KO mice. The underlying mechanism of the testicular dysfunction is remained to be undetermined. Weight of the testes in AhR-KO mice is generally smaller than those in wild-type mice (75.2 ± 4.2, 84.9 ± 8.1, 105.5 ± 1.4 mg; homozygous AhR-KO, hemizygous AhR-KO, and wild-type mice, respectively). Possible fictional differences of their testicular glands may be more evident in AhR-KO mice treated with MNU (50 mg/kg body weight) than in nontreated mice. The testicular body ratio after MNU treatment in wild-type mice show a 91.0% decrease in wild-type mice compared with the control without MNU treatment, whereas the ratio after MNU treatment in AhR-KO mice show a 71.4% decrease compared with the same control wild-type mice without MNU treatment. These results indicate that the testicular tissue of AhR-KO mice shows a dysfunction in cellular proliferation and regeneration during the course of the development and the recovery after tissue injury.

Nebert et al. also found a low fertility in the D2-linked DBA strain, which seems to be linked to AhR affinity [60]. Thus, AhR seems to contribute to the stabilization of fertilization, which might be one of the reasons its gene has diversified across species during the course of its evolution. Because the focus of this section is the hematopoietic system, further description on the functional contribution of AhR to fertilization may be discussed elsewhere.

2.3. *$AhR^{-/-}$ mice show earlier onset of spontaneous neoplasms—Gompertzean accelerated aging*

Fig. 1 shows the incidence of spontaneous malignant lymphomas in each genotype group for *AhR* plotted along the ordinate axis vs. age in days plotted along the horizontal axis. The development of lymphomas in the $AhR^{-/-}$ group (darkly shaded columns) starts earlier than that in the $AhR^{+/-}$ (lightly shaded columns) and wild-type (open columns) groups. The incidence

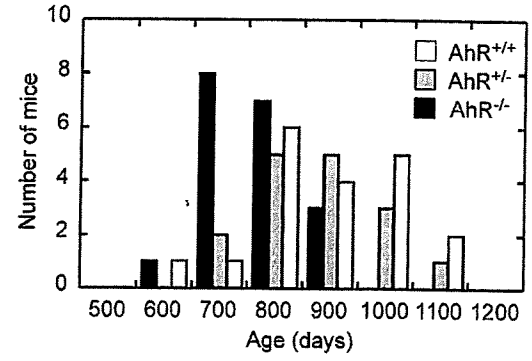

Fig. 1 – Incidences of spontaneous malignant lymphomas at unit time intervals for wild-type ($AhR^{+/+}$) mice and AhR-deficient ($AhR^{-/-}$, $AhR^{+/-}$) mice. (Open columns, $AhR^{+/+}$; lightly shaded columns, $AhR^{+/-}$; darkly shaded columns, $AhR^{-/-}$ groups.)

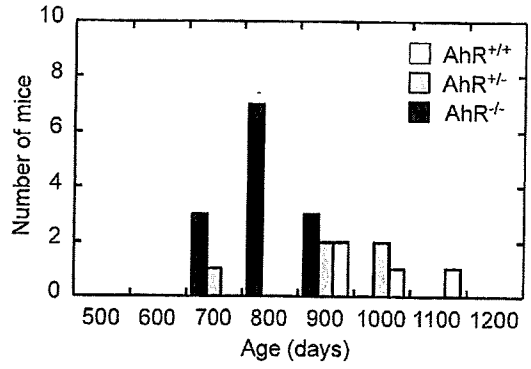

Fig. 2 – Incidences of spontaneous hepatomas at unit time intervals in wild-type ($AhR^{+/+}$) mice and AhR-deficient ($AhR^{-/-}$, $AhR^{+/-}$) mice. (Open columns, $AhR^{+/+}$; lightly shaded columns, $AhR^{+/-}$; darkly shaded columns, $AhR^{-/-}$ groups.)

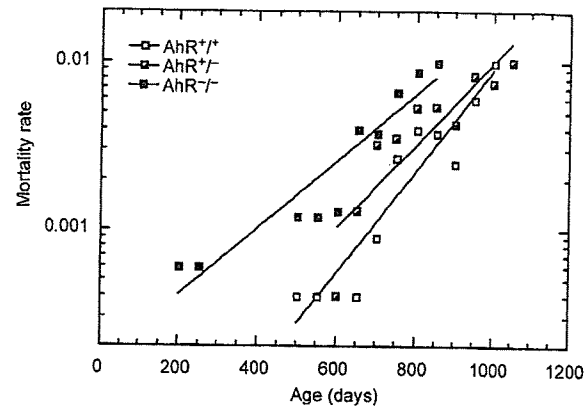

Fig. 3 – Gompertzean expressions [61] of mortality rates of mice of different genotypes: wild-type ($AhR^{+/+}$) mice and AhR-deficient ($AhR^{-/-}$, $AhR^{+/-}$). (Open squares, $AhR^{+/+}$; half-closed squares, $AhR^{+/-}$; closed squares, $AhR^{-/-}$ groups.)

of lymphomas in the $AhR^{-/-}$group peaked at 700 days old, whereas those in the $AhR^{+/-}$ and wild-type groups peaked at 850 and 1000 days old, respectively. Similarly, the incidences of spontaneous hepatomas in each genotype group are plotted in Fig. 2. In this figure, spontaneous hepatomas in the $AhR^{-/-}$ group appear at 700 days old and those in the wild-type group appear much later (900 days) at significantly lower incidences. In Fig. 3, the mortality rate/unit time interval for each genotype group is plotted. Mortality rate/unit time interval is shown in the ordinate on a logarithmic scale and age in days is plotted along the horizontal scale (Gompertzean expression [61]). In this figure, the line for closed squares for the $AhR^{-/-}$ mice shows a much early onset curve with a lower and flatter slope than the line for open square for the wild-type group. Thus, the mortality rate of the $AhR^{-/-}$ group can be concluded to indicate "accelerated aging". The shortened lifespan observed in the AhR-KO mice may be due to the impairment of a possible suppression gene in the KO mice. However, some mice for each survival curve are non-tumor-bearing mice. The mechanism of this accelerated aging may not be as simple as that involving a tumor suppressor and remains to be elucidated.

AhR is an orphan receptor whose original physiological function remains unclarified. Since AhR-KO mice were found to show an earlier onset of spontaneous neoplasms than wild-type mice, AhR was assumed to play a suppressor gene function [62]. However, because not all AhR-KO ($AhR^{-/-}$) mice or wild-type mice die of spontaneous neoplasms, the function of wild-type AhR may also be associated with a possible genomic stabilization, consequently extending the lifespan of mice simultaneously. What are the underlying mechanisms that contribute to the extended longevity? Evaluation of reactive oxygen species (ROS) using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) dye (Fig. 4) showed a prominent increase in oxidative stress in unfractionated bone marrow cells as well as in hematopoietic progenitor cells in AhR-KO mice [63] (Fig. 5). Hematopoietic progenitor cells are quiescent in anoxic environment, and are regulated by a weak oxidative stimulation as redox homeostatic regulation [64]. Thus, the reactivity of the fraction to the DCFH-DA dye was higher in AhR-KO mice than in wild-type mice, which is in good agreement with the mechanism underlying genomic stabilization under a

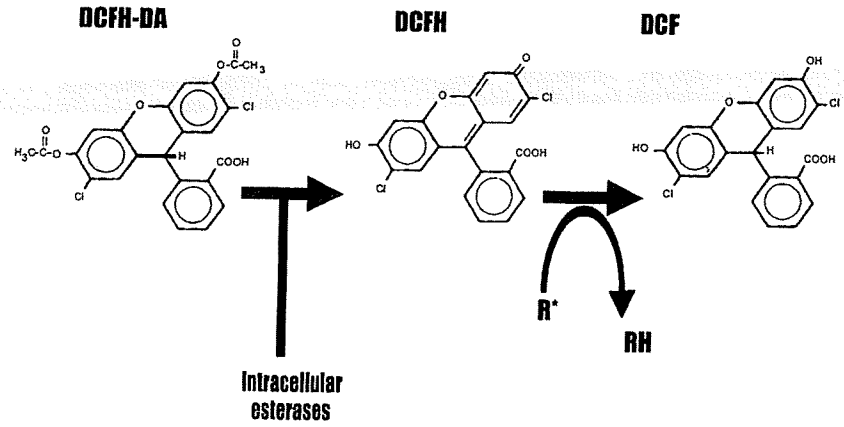

Fig. 4 – 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) as ROS indicator [92] (DCFH: 2′,7′-dichlorodihydrofluorescein, DCF, 2′,7′-dichlorofluorescein).

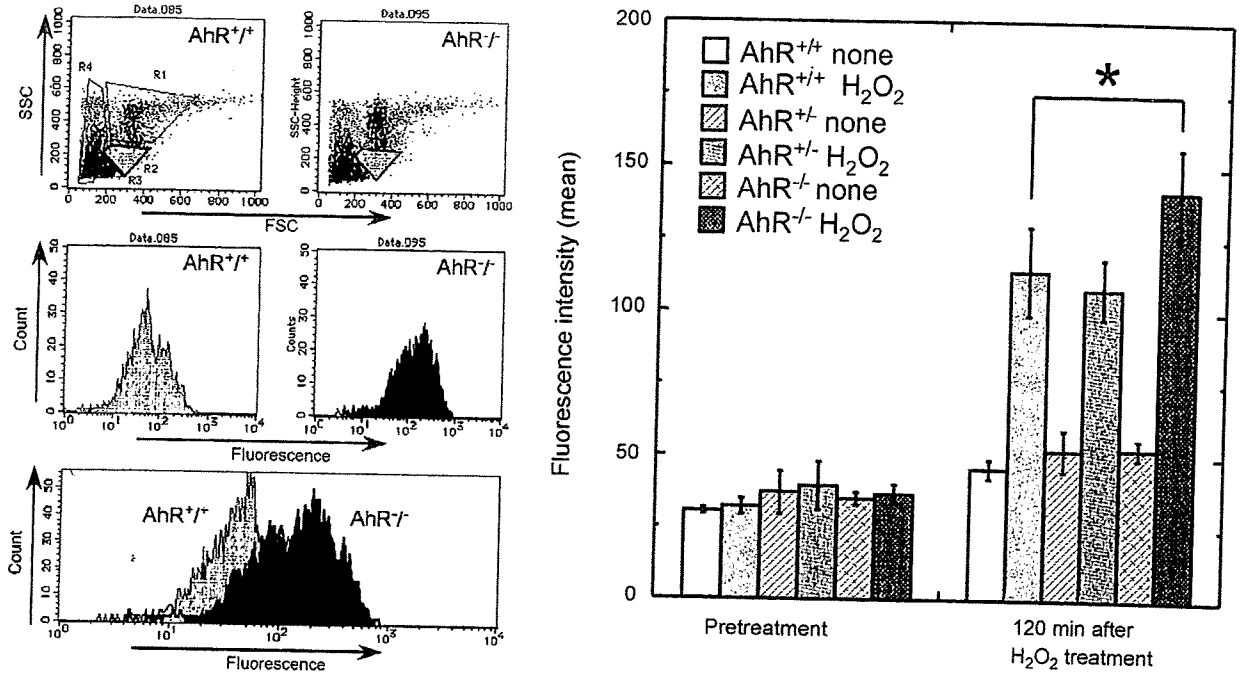

Fig. 5 – Fluorescence intensities of DCF dye in primitive bone-marrow cell fraction between the steady state (none) and the H_2O_2 treatment. Left panel, top row: primitive progenitor cell compartment (R2) were sorted out from bone marrow (BM) cells of wild-type ($AhR^{+/+}$) mice (left) and AhR-KO ($AhR^{-/-}$) mice (right) using a cell sorter as displayed between the forward scatter (horizontal axis) and the side scatter (ordinate axis). Left panel, middle and bottom rows: Relative cellular counts of the hematopoietic progenitor cells, ordinate axis vs. DCF dye-fluorescence intensity, horizontal axis, are compared between $AhR^{+/+}$ mice (middle left) and $AhR^{-/-}$ mice (middle right). Both profiles are compared in the bottom. Right panel: Fluorescence intensity of DCF dye of primitive hematopoietic progenitor fractions (R2 area) before (pre) and after (H_2O_2) treatment. Fluorescence intensity of DCF dye in R2: Groups for H_2O_2 treatment at 0 min on the left bars ($AhR^{+/+}$, none and H_2O_2; $AhR^{+/-}$, none and H_2O_2; $AhR^{-/-}$, none and H_2O_2, respectively), and Groups for H_2O_2 treatment at 120 min on the right bars ($AhR^{+/+}$, none and H_2O_2; $AhR^{+/-}$, none and H_2O_2; $AhR^{-/-}$, none and H_2O_2, respectively).

low oxidative tension in combination with the suppressor gene function and the consequent longevity observed in wild-type mice.

2.4. *Longevity as essential driving force for evolution of AhR*

Different mouse strains in terms of aryl hydrocarbon receptor function, receptor concentrations, and lifespans are compared. Because mice of different strains show different spontaneous neoplastic propensities, sometimes possess different AhR structures, and have different lifespans, available databases for such AhR-related functional parameters were compared. The results obtained are shown in Table 2.

In this table, two strains, namely, C3H/He and DBA/2, showed a low affinity or a low enzyme activity for constitutively activated cytochrome P450 (CYP) 1A2, whereas C57BL/6J, showed a high affinity or a high enzymatic activity for CYP1A2

Table 2 – AhR binding affinities and receptor activities, cell cycles, and life spans among murine species.

Strain	AhR affinity[a]	CYP1A2 enzyme activity[b]	Receptor concentration[c]	Cell cycle[d]	Lifespan[e] (days)	Notes
C3H/He	High*	Low to mid	86 ± 23	High	500	*Low signal induction
DBA/2J	Low	Low	–	High	710*	*708.7 days in other litr.[e]
C57BL/6J	High	High	151 ± 26	Low	789*	*860.8 days in other litr.[e]

[a] Murine Ah receptor specified by the Ah^d and Ah^{b-3} alleles is compared.
[b] Activities for methoxyresorufin o-demethylation (MOD) and pentoxyresorufin o-dealkylation, and metabolic activation of IQ for phenobarbital were tested. For activity in DBA/2J, high in male, but low in female for CYP1A2. Activity for MOD was significantly low for both genders [65].
[c] Murine Ah locus (mg/protein) [66].
[d] Scored using hematopoietic cobblestone area-forming cell assay [67].
[e] Data cited from Van Zant and de Haan [67] and Forster MJ et al. [68].

[65]. Although C3H/He mice showed a high affinity, the receptor concentration (mg/protein) measured was low [66]; thus, the total activity reported in the literature is low. It is interesting to determine correlations between AhR activity and the stage of cell cycle or lifespan, because mice with a high AhR activity, i.e., C57BL/6J, seem to show a suppressed cell cycle and longer lifespan, whereas mice with a low AhR activity, i.e., C3H/He, seem to show an accelerated cell cycle with a shortened lifespan [67,68]. No comparable data on genomic stability (or instability) fully supports the above-mentioned AhR activity are available. However, the correlations between AhR activity and the stage of cell cycle or lifespan seem to be plausible and compatible with the results of our present study of experimental induction of AhR-deficiencies.

We also found that AhR-KO mice show an earlier onset of spontaneous neoplasms than wild-type mice [62]. Thus, it is plausible that *AhR* functions as a tumor suppressor gene in the steady state. Furthermore, because not all of these mice die of malignant neoplasms, AhR may also extend the lifespan of these mice, i.e., it has "longevity" function (Fig. 6). Such biological plausible functions are possible reasons for the molecular evolution of *AhR* from homologues in invertebrates, such as nematodes, equivalent to those in vertebrates. However, one question remains: Why do AhR-KO mice show early onset of spontaneous neoplasms? Supposedly these mice should exhibit unfavorable xenobiotic responses when AhR is knocked out [69]. Furthermore, the mechanisms of the possible suppressive function of wild-type *AhR* remain to be elucidated.

Successful fertilization, tumor suppression, and longevity seem to be essential driving forces of phylogenic evolution of AhR in vertebrate species or, if not all, at least in mammalian species. Not many similar reports are available in the literature but some of them are strongly linked to the present issue. According to Abbott [70], the concentrations of AhR after birth increased rapidly and plateaued from 2 to 21 days and then rapidly decreased in the liver and lungs. Nebert et al. also discussed about the possible AhR-mediated longevity but the lifespan of experimental mice is too short to speculate on longevity [60]. This was supported by a study by Spindler et al. in 1989, which showed distinct differences in the maximum lifespan among different mouse strains with different induction levels of cytochrome p450s [71]. These previous studies suggest the functional advantages of AhR expression, but the mechanism underlying these functional advantages are poorly described. These are much strongly related to the localization of AhR expression in the tissue and AhR-mediated cell-cycle regulation. Findings possibly related to these issues will be described later.

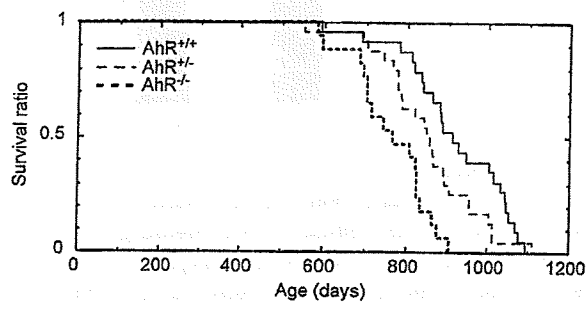

Fig. 6 – Survival curves for wild-type ($AhR^{+/+}$) mice and AhR-deficient ($AhR^{-/-}$, $AhR^{+/-}$) mice. (Solid line, $AhR^{+/+}$; long dotted line, $AhR^{+/-}$; short dotted line, $AhR^{-/-}$ groups.)

3. Hematopoietic progenitor cells and their cell-cycle regulation

As discussed in the previous section, although we know that AhR functions in maintaining lifespan, we do not know its underlying mechanism. In this particular section, we focus on the biological function of AhR in hematopoietic progenitor cells and in their cell-cycle regulation to elucidate the relevant mechanism that may explain such advantages of the function of AhR.

The hematopoietic microenvironment is hypoxic [72,73], which is located beneath the periosteal region and forms niches. The niches are thought to consist of genes, such as N-cadherin [19–21], Jagged1/Notch [22], Angi/Tie-2 [23], osteopontin [24], and SDF1(CXCL12)/CXCR4 [25,26] genes, and possibly also the connexin 32 [17,27] gene, which maintains dormancy of hematopoietic stem/progenitor cells, because of the hypoxic state located distant from the vascular network. Stromal cells and endosteal cells in the hypoxic state may express hypoxia inducible factor (Hif)-1alpha [74], and also in some cases, HIF-2alpha [75] is expressed by hematopoietic stem/progenitor cells themselves, which may interact with ARNT molecules leading to steady-state hematopoiesis.

Regarding xenobiotic responses induced by AhR, hematopoiesis is often suppressed probably because of a possible recruitment of ARNT molecules to activate AhR. The monumental study by Luster in 1985 [76] showed a decrease in CFUs following the exposure of mice to TCDD, which may be based on this mechanism. AhR signaling also induces cell-cycle suppression, the mechanism of which has not been elucidated. Nevertheless, such deceleration of cell cycle in hematopoietic stem/progenitor cells contributes to longer stem cell survival, which may be the reason for the extension of animal lifespan.

3.1. *Aryl hydrocarbon receptor regulates hematopoietic progenitors*

When AhR is activated by xenobiotic ligands, ligand-specific transcription induces the production of drug-metabolizing enzymes, Cyp1A1 and 1B1, as the major products [1,2], which in turn induce oxidative stresses [6,77] and the consequent hematopoietic impairment by the up-regulation of cyclin-dependent kinase inhibitors [78,79]. Such hematopoietic impairment in stem-cell-specific cell cycling can be evaluated by the BUUV method [27,80], which is described in Section 3.3. Under xenobiotic stimulation with AhR ligands, a slight inhibition of transportation of ARNT required by Hif-1alpha and Hif-2alpha in the hypoxic hematopoietic microenvironment induces a possible inhibition of primitive hematopoiesis. On the other hand, steady-state expression of AhR makes ARNT available for Hif-1alpha and Hif-2alpha, the up-regulation of which is maintained by the relatively hypoxic hematopoietic microenvironment. Thus, AhR is important because ARNT released from AhR functions in transcription of

various hematopoietic factors, such as erythropoietin, CXCR4 [81], SDF-1 [73], vascular endothelial growth factor (VEGF) [82], and VE-cadherin [83]. According to the study by Adelman et al. on ARNT-KO mice, production of various CFUs derived from hematopoietic progenitors from embryonic stem (ES) cells markedly decreases in a ARNT-gene-dosage-dependent manner [84]. ARNT is, thus, important to maintain such primitive hematopoiesis.

3.2. *Aryl hydrocarbon receptors and cell cycle*

Whether AhR physiologically suppresses cell growth through relevant signals via a possible endogenous ligand [85] or facilitates cell-cycle progression from G_1 with *Fos* and *Jun* signaling, remains controversial [86]. In the authors' previous study, authors attempted to elucidate a possible hidden function of AhR in hematopoiesis using AhR-knockout mice [87].

3.2.1. *Aryl hydrocarbon receptor and B cell progenitors*

As the authors focused on B cell suppression during B lymphopoiesis [5], the effects of TCDD exposure on hemopoiesis were extensively investigated, since the inhibitory effects of TCDD on bone marrow and immunological parameters, including granulocyte-macrophage (GM) colony forming unit (CFU) and other progenitors, were first recognized by Luster and coworkers in the early 1980s [76,88]. The down-regulation of AhR expression attenuates myelosuppression in thioredoxin (Trx)-overexpressing mice, as determined by hemopoietic colonization assay, which elucidated the linkage of AhR signals to the antioxidant cascade induced by reactive oxygen species, ROS, after TCDD exposure [89,90].

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

3.2.2. *$AhR^{-/-}$ mouse shows significant enhancement of hematopoiesis*

The number of WBCs increases in $AhR^{-/-}$ mice (Fig. 7(a)). This is the first observation in $AhR^{-/-}$ mice [91] that is consistent with the hypothetical description by Adachi et al. [85] in which a possible physiological ligand is speculated to suppress hema-

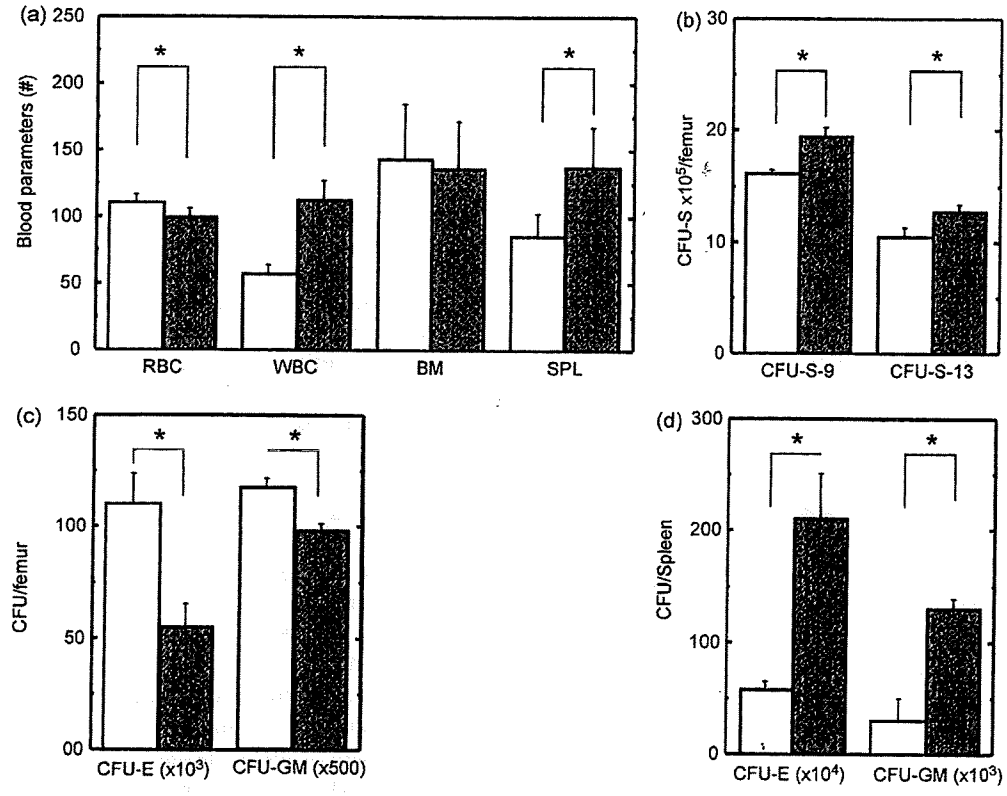

Fig. 7 – Comparison of various blood parameters between wild-type mice (open columns) and AhR-KO ($AhR^{-/-}$) mice (shaded columns); (a) peripheral blood, bone marrow (BM) and spleen weight. #: Vertical axis "Parameters" indicate the counts of peripheral red blood cells (RBCs, $\times 10^8$/ml) and white blood cells (WBCs, $\times 10^6$/ml), BM cellularity ($\times 10^5$/femur), and weight of the spleen (SPL, mg). (b) Number of colony forming units in spleen (CFU-S, $\times 10^5$/femur) observed on days 9 (CFU-S-9) and 13 (CFU-S-13). (c) Numbers of in vitro granulocyte-macrophage CFUs (CFU-GM, ×500/femur) and erythroid CFU (CFU-E, $\times 10^3$/femur) in femoral BM. (d) Numbers of CFU-GM ($\times 10^3$/spleen) and CFU-E ($\times 10^4$/spleen) in spleen.
***: Significant difference between wild-type and AhR-KO mice determined by t-test at $p < 0.05$.**

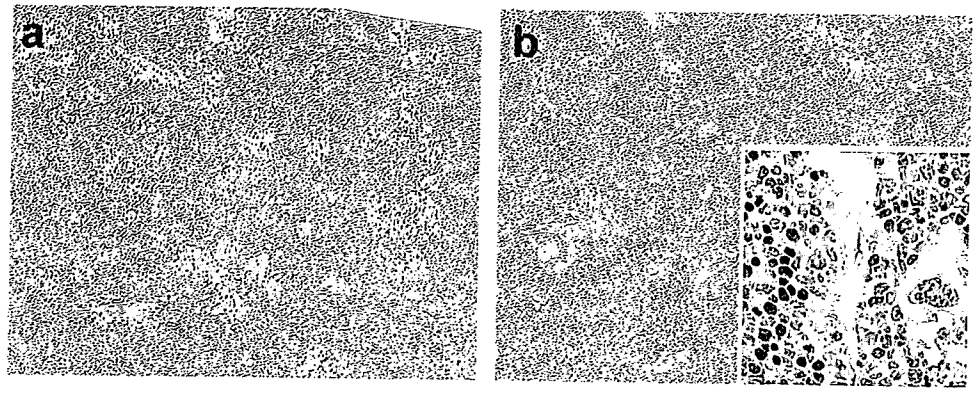

Fig. 8 – Histopathological findings in spleen from wild-type mice (a) and AhR-KO ($AhR^{-/-}$) mice (b). Note, a prominent enhancement of hemopoiesis in the spleen can be observed in $AhR^{-/-}$ mice (b). (HE staining. Magnification: a and b ×20, inset of b ×80.)

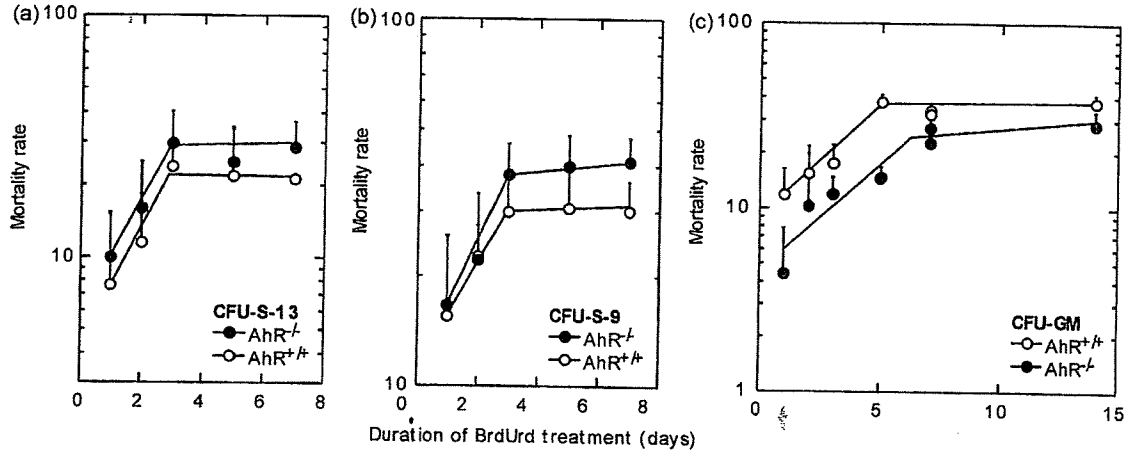

Fig. 9 – Hemopoietic progenitor cell kinetics of each hemopoietic progenitor compartment from wild-type mice ($AhR^{+/+}$) (open symbols) and AhR-KO ($AhR^{-/-}$) (closed symbols), measured by BUUV method [32,80]; numbers of colony forming units in spleen observed on days 13 (CFU-S-13) (a) and 9 (CFU-S-9) (b), and numbers of granulocyte-macrophage CFU (CFU-GM) (c). CFU-S-13 and CFU-GM are significantly different as determined by a paired t-test between $AhR^{+/+}$ and $AhR^{-/-}$ mice ($p < 0.05$). The plateau level (between day 3 and day 7 of BrdUrd treatment) of CFU-S-9 is also significantly different, as determined by the t-test between $AhR^{+/+}$ and $AhR^{-/-}$ mice ($p < 0.05$)).

topoiesis in $AhR^{+/+}$ mice. This is also consistent with the higher numbers of myeloid progenitor cells, i.e., CFU-S-9 and CFU-S-13, observed in $AhR^{-/-}$ mice (Fig. 7(b)). Thus, steady-state hematopoiesis is presumed to be suppressed via AhR signaling by a possible physiological ligand, which is as yet not identified in $AhR^{+/+}$ mice. In response to such an AhR-null effect, $AhR^{-/-}$ mice reversely show extensive hematopoiesis in the spleen (Fig. 8(b)) as compared with wild-type mice (Fig. 8(a)), although this hemopoietic enhancement is also reflected in another negative hemopoietic regulation in the bone marrow.

3.2.3. *AhR promotes cell cycling in hemopoietic progenitors*

Interestingly, when bone marrow cells are removed from $AhR^{-/-}$ mice and $AhR^{+/+}$ mice, and are grown in *in vitro* colony assay, the numbers of CFU-GM and CFU-E are both significantly lower in $AhR^{-/-}$ mice (Fig. 7(c)), implying that AhR signaling promotes the acute phase response to cytokines during colony growth. The decrease in the number of CFU-E, as shown in Fig. 7(c), possibly affects the number of RBCs in the peripheral blood of $AhR^{-/-}$ mice (Fig. 7(a)). These observations are consistent with those for other hemopoietic progenitors (data not shown). Interestingly, in response to such an AhR-null effect, AhR-KO mice in contrast show extensive hemopoiesis in the spleen (Fig. 7(d)), which results in a significant increase in spleen weight (Fig. 7(a); rightmost) [28,91].

3.3. *Cell kinetics of CFU-GM receives negative feedback in steady state*

The BUUV method[1] shows a clear enhancement of the cell cycle in primitive progenitor cells, CFU-S-13 (Fig. 9(a)), and in

[1] BUUV method is to evaluate hematopoietic stem/progenitor cell-specific kinetics by continuous perfusion of 5-bromo-2′-deoxyuridine (BrdUrd) through an osmotic pump (Alza Corp., Palo Alto, CA) followed by ultraviolet (UV) – A exposure and hematopoietic colonization assay, which permits to obtain a variety of parameters in the cell kinetics of the hemopoietic progenitor cell compartment, such as a doubling time, a size of cycling- or quiescent fraction, and also the size of cycling fraction during the unit time interval.

relatively mature progenitor cells, CFU-S-9 (Fig. 9(b)) in $AhR^{-/-}$ mice. Although the precise mechanism underlying this phenomenon is not clarified yet, the cell kinetics of CFU-GM becomes suppressed in terms of percent cycling fraction per unit time, i.e., less than 5% (Fig. 9(c)), which may be due to a possible negative feedback to an up-regulated cell kinetics of primitive progenitors (Fig. 9(a) and (b)).

The lack of AhR and the complex compensation of bone marrow hematopoiesis might still be insufficient in $AhR^{+/+}$ mice, because a compensatory increase in splenic weight in $AhR^{-/-}$ mice is evident (Figs. 7(a) and 8). As reported by Puga et al. [86], AhR functions as a cell-cycle regulator rather than as a drug-metabolizing enzyme inducer; thus, possible phenotypes transmitted via AhR may be diversified.

4. Hematopoietic progenitor cells and xenobiotic responses

By analyzing antioxidative responses to thioredoxin concerns, we have recently found that benzene-induced xenobiotic responses are associated with antioxidative signaling [92]. Thus, the increased incidence of spontaneous neoplasms and accelerated aging observed in AhR-KO mice can be hypothesized as consequences of genomic instability possibly due to the absence of xenobiotic or antioxidative responses. ROSs in hematopoietic tissues in both AhR-KO and wild-type mice were evaluated using the DCFH-DA dye by flow cytometry, followed by the fractionation of hematopoietic progenitor cells. Hematopoietic tissues from $AhR^{-/-}$ mice showed a high reactivity to DCFH-DA, as shown in Fig. 5. Because the AhR expression level is high on primitive hematopoietic progenitor cells [91] fractionated primitive hematopoietic progenitor cells were also evaluated for their reactivity to DCFH-DA, which was found to be higher than the reactivity of other unfractionated bone marrow cells. Hematopoietic progenitor cells, i.e., the LKS fraction, are quiescent in an anoxic environment; furthermore, its activity is regulated by weak oxidative tension. Thus, the higher reactivity of the hematopoietic progenitor cell fraction in $AhR^{-/-}$ mice to the DCFH-DA dye is considered to be in good agreement with the underlying mechanism of genomic stability of wild-type $AhR^{+/+}$ mice, which may be linked to the suppressive function of AhR and the consequent longevity.

4.1. *Aryl-hydrocarbon-receptor-mediated hematopoietic alteration by xenobiotic substances*

When xenobiotic ligands such as TCDD and/or benzo[*a*]pyrene are applied to AhRs, transcription of drug-metabolizing enzymes such as Cyp1A1 and 1B1 are induced [1,2]. The consequent induction of oxidative stress by these induced Cyp1A1 and 1B1 is known precisely [6,77]. The function of AhR in relation to the hematopoietic system involves two possible factors for hematopoiesis. First, induction of Cyp1A1 and 1B1 by xenobiotic responses consequently induces up-regulation of a cyclin-dependent kinase inhibitor, $p27^{kip1}$ or $p21^{waf1}$, which readily suppresses the cell cycle [78,79]. Benzene is a unique newly found chemical whose toxicity is mediated by AhR [69], which suppresses the hematopoietic stem-cell-specific cell cycle [93], which will be introduced as a model of hematopoietic stem cell modulation in the next section.

Second, what happens in the primitive hematopoietic stem cell system when AhR is stimulated by ligand-dependent up-regulation? In this situation, ARNT required by Hif-1alpha and Hif-2alpha in the hypoxic hematopoietic microenvironment is considered to inhibit primitive hematopoiesis [78]. Because of this suppression of primitive hematopoiesis, various hematopoietic progenitor cell compartments are decreased in number, as observed by Luster et al. in a variety of CFUs after exposure of mice to TCDD [76,88].

The above-mentioned hematopoietic impairment is based on the impaired function of the long-term reconstitution activity of the LKS fraction, i.e., primitive hematopoietic progenitor compartment. The long-term reconstitution activity of the LKS fraction repopulated in lethal-dose-irradiated recipients was heavily impaired after TCDD exposure. When repopulation of the LKS fraction is applied following TCDD exposure, while the nontreated LKS fraction successfully reaches a chimeric ratio of 80% with respect to the competitive donor fraction by 140 days after transplantation, the chimeric ratio is limited only to 15% with peak at 80 days after transplantation and the LKS fraction exposed to TCDD disappears by 140 days after transplantation [94].

Similar lymphopoietic alterations are observed after exposure to TCDD such as inhibition of thymic cell development [95], dysregulation of regulatory T and pro-inflammatory T cell differentiation [96], alteration of B cell maturation and CD34-positive human hematopoietic progenitor cells [97]. These alterations are also observed for ligands other than TCDD [96,98].

4.2. *Aryl-hydrocarbon-receptor-mediated benzene hematotoxicities*

Recent studies have shown that AhR in primitive cells transmits negative signals for the proliferation of such cells [91]. As we previously reported, primitive hemopoietic progenitor cells increases in number in AhR-KO mice; on the other hand, relatively mature progenitor cells, decreases in number in a homeostatic manner [91].

We have reported that benzene-induced hemopoietic toxicity is transmitted by AhR [69]. We also found that CYP2E1 related to benzene metabolism is also up-regulated in the bone marrow following benzene exposure [99]. Therefore, it is of interest to hypothesize a greater role of bone marrow cells in hemopoietic toxicity than in hepatic metabolism. Accordingly, in our present study, benzene-induced hemopoietic toxicity was evaluated in wild-type mice after a lethal dose of whole-body irradiation followed by repopulation with bone marrow cells that lack AhR or, *vice versa*, in AhR-KO mice after repopulation with wild-type bone marrow cells. As a result, benzene-induced hemopoietic toxicity seems to have been transmitted through AhR, and benzene was transformed by *de novo* metabolism with CYP2E1 in the bone marrow.

Six weeks after the repopulation, the steady-state hematopoietic parameters for repopulated mice were obtained and are shown in Fig. 10. The results were essentially the same between the mice repopulated with wild-type bone marrow

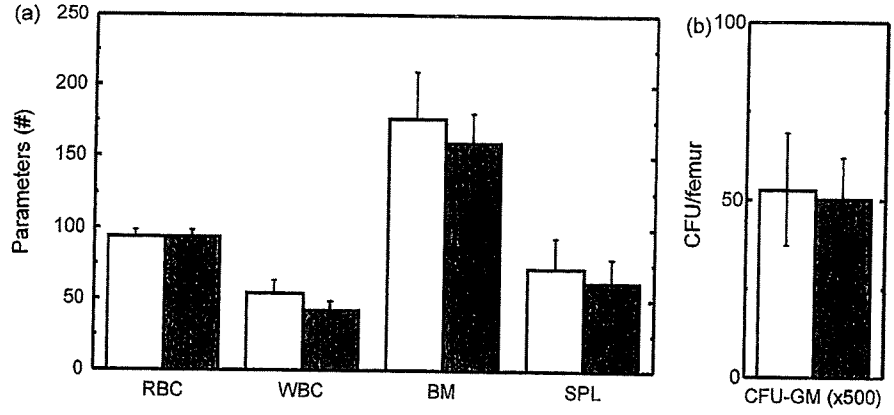

Fig. 10 – Comparison of various blood parameters between mice repopulated with wild-type BM (open columns) and AhR-KO ($AhR^{-/-}$) BM (shaded columns) cells; (a) peripheral blood, BM and spleen weight. #: Vertical axis "Parameters" indicates the counts of peripheral RBCs ($\times 10^8$/ml) and WBCs ($\times 10^6$/ml), BM cellularity ($\times 10^5$/femur), and weight of spleen (SPL, mg). (b) Numbers of CFU-GM (5×10^2/femur) per femur.

cells (open columns) and those repopulated with AhR-KO bone marrow cells (shaded columns).

Fig. 11(a) and (b) show the percentages of RBCs (a) and WBCs (b) with respect to that of the control in the peripheral blood after the repopulation with bone marrow cells. In the wild-type mice repopulated with wild-type bone marrow cells and those with AhR-KO bone marrow cells (open and closed symbols, respectively), benzene exposure induced a slight but statistically significant decrease in RBC count compared with the sham exposure except on day 5 in the wild-type group (100% with standard deviation of the mean indicated by horizontal lines: Fig. 11(a)). The dose used in our present study was sufficiently high, and the decrease in RBC count was readily observed within two weeks of exposure.

The decreases in WBC count shown in Fig. 11(b) are more significant than those in RBC count throughout the exposure period except on day 5 in the AhR-KO group (the data were significantly different between wild-type mice (50.8 ± 11.2%) and AhR-KO mice (70.6 ± 17.6%; $p = 0.024$)).

As shown in Fig. 11(c), the decrease in the number of bone marrow cells after benzene exposure is significant in the mice repopulated with wild-type bone marrow cells specifically on

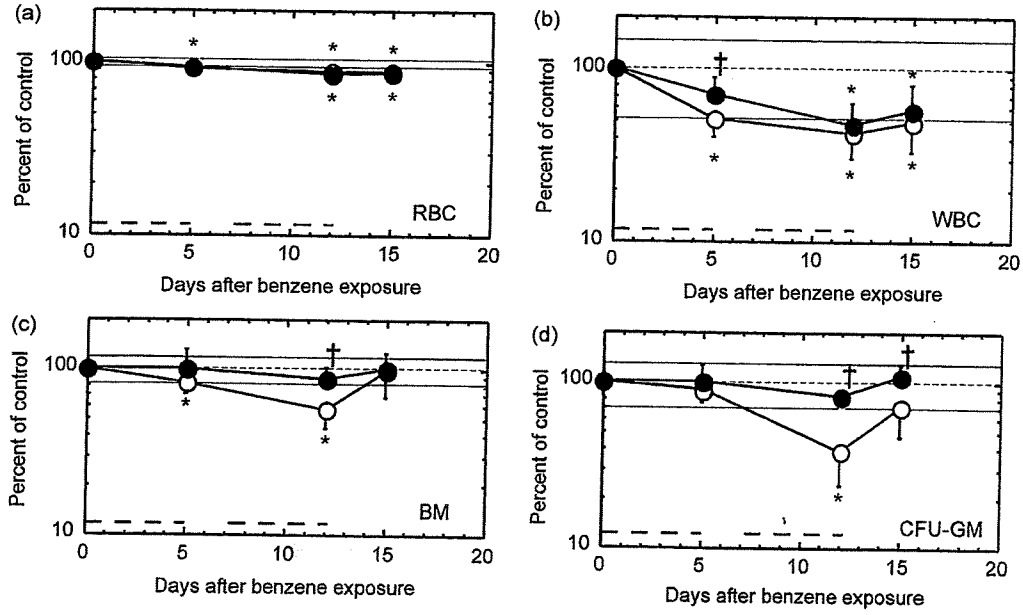

Fig. 11 – Changes in percentage of RBCs (a), WBCs (b), BM cells (c) and CFU-GM (d) of mice repopulated with wild-type BM (open symbols) and AhR-KO ($AhR^{-/-}$) BM (closed symbols) cells during and after benzene exposure, with respect to those in each sham-exposure group. Vertical bars indicate the standard deviation of the mean. Horizontal dashed line indicates the mean (100%) and the solid lines indicate the standard deviation of the mean (100%) from the sham-exposure control wild-type group. The dashed line at the bottom indicates benzene exposure duration.
***: Significant difference between sham-exposure group and benzene-exposed group determined by t-test at $p < 0.05$.**
†: Significant difference between wild-type mice and AhR-KO mice.

days 5 and 12 (82.2 ± 12.0%, $p = 0.035$ and 65.4 ± 20.3%, $p = 0.007$, respectively; number of cells obtained on day 12, significantly different between the wild-type mice and the AhR-KO mice (75.4 ± 19.7%; $p = 0.014$)), which returned to the normal range by day 15, that is, 3 days after cessation of benzene treatment. In contrast to the peripheral blood parameters (Fig. 11(a) and (b)), the number of bone marrow cells in the mice repopulated with AhR-KO bone marrow cells did not show any decrease, but the mice showed a clear nullification of benzene-induced decrease in the number of bone marrow cells (86.7 ± 14.9%; $p = 0.057$). Concerning the weight of the spleen, there are no significant differences among the groups regardless of the duration of benzene treatment and AhR expression level (not shown).

In Fig. 11(d), the number of CFU-GM in the bone marrow of mice repopulated with wild-type bone marrow cells much more significantly decreased on day 12 (open symbols, 37.8 ± 14.2%, $p = 0.019$; number of cells, significantly different between wild-type mice and AhR-KO mice (82.0 ± 7.0%; $p = 0.001$)), which rapidly returned to the normal range by day 15, 3 days after cessation of benzene treatment. As shown in this figure, interestingly, the benzene-induced decrease in the number of CFU-GM in the bone marrow of mice repopulated with the AhR-KO bone marrow cells (closed symbols) is clearly nullified for the wild-type bone marrow cells (open symbols), and the number remains within the range found for the sham exposure. The reason for this very prominent decrease in the number of CFU-GM observed in the case of benzene exposure may be due, in part, to the expression of AhR, whose level is significantly high in primitive hematopoietic progenitor cells [5,100]. The KO of AhR nullified the decrease in the number of CFU-GM much more significantly than the decrease in peripheral blood parameters.

5. Summary

Oxidative stress induced by AhR-mediated benzene metabolites induces xenobiotic hematological malignancies in C57BL/6 mice. The encounter of AhR with benzene may not be the original biological relevance of historic and phylogenic evolution of the AhR molecule in a wide range of animals from invertebrates to vertebrates including humans. Would not this be an ironical encounter?

In experimental mice, the existence of AhR apparently extends their lifespan as compared with AhR-KO mice (mean life spans, 756 days in AhR-homozygous KO and 890 days in wild-type C57BL/6). The major reasons for this extension of lifespan by AhRs seem to be the suppression of epigenetic tumorigenicity and age-related gerontological diseases, possibly owing to the advantages derived in reducing oxidative stresses through the AhR function. No precise mechanism has been reported, but the onset of spontaneous neoplasms and non-neoplastic senescent diseases is delayed in wild-type mice as compared with that in AhR-deficient mice. Furthermore, when AhR is knocked out, in the case of the hematopoietic system, the capacity to maintain the hematopoietic stem cell compartment is clearly diminished and the major fraction tends to differentiate into descendant blood cell classes. The hematopoietic stem cell compartment shows the following. First, the compartment shows a diminished capacity to maintain the LKS fraction, the stem cell compartment, as observed by cell sorting analysis. Second, it shows a decrease in the fraction of the dormant stem cell/progenitor compartment, as measured by the BUUV method for evaluating kinetics of hematopoietic progenitor cells, by continuous in vivo treatment with BrdUrd and BrdUrd-labeled cell-purging with ultraviolet (UV) light. Thus, the possible regulation of hematopoietic stem cells maintaining the fraction of dormant hematopoietic stem cells may have mechanistic relevance in terms of a possible genomic stabilization by AhR, which should be elucidated.

The hematopoietic microenvironment is hypoxic during the steady state. This hypoxic state induces AhR to release (unbound) ARNT to Hif-1alpha as well as Hif-2alpha; thus, the function of slight changes in regulating oxidative stress for a hematopoietic trigger in the hematopoietic microenvironment in wild-type mice may not be markedly different from that in the AhR-KO mice. However, when a change induces a much higher extent of oxidative stress induced by substances such as hydrogen peroxide in AhR-KO mice, the increased amount of ROSs is readily detected in AhR-KO mice, whereas wild-type mice show active removal of oxidative stress by ROS-scavenging molecules, resulting in notable differences in the amount of ROSs between wild-type and AhR-KO mice (Fig. 5).

Evidence was found in the liver that the expression of antioxidative stress genes such as superoxide dismutase 1 (Cu/Zn-SOD) and SOD2 (Mn-SOD) genes, as well as the Trx gene, which is located downstream of AhR and carrying XRE, are more highly expressed in wild-type mice than in AhR-KO mice [63].

In contrast to the above-mentioned beneficial biological function of AhR, in the case of benzene exposure, benzene exposure induces hematopoietic disturbances and the consequent leukemias. This seems to be induced by oxidative stress in AhR wild-type mice because Trx-overexpressing mice in contrast show nullification of hematopoietic disturbances owing to oxidative stress removal. Here is an enigma of benzene-induced hematotoxicity, that is, oxidative stress induced by as low as one part per million of benzene, which cannot be removed by antioxidative molecules associated with AhR as compared with those induced by hydrogen peroxide. On the other hand, the benzene exposure of AhR-KO mice is supposed to show none of the benzene-mediated oxidative-stress-induced hematopoietic disturbances or myeloid leukemias, simply owing to nullification of benzene metabolism in these mice lacking AhR.

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Future alternatives in "3Rs": Learning from history

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Abstract
A large decrease in the number of experimental animals utilized in testing and research was reported in the last decade (Surveyed by Expt'l Animal Soc.[1]). For rats, the numbers used in experiments in Japan were 2.09 million in 1995, 1.53 million in 1998, and 1.24 million in 2001. Thus, there was a 40% decrease in the number of rats used from 1995 to 2001. For mice, a larger decrease (58%) was also observed, from 6.68 million in 1995 to 2.80 million in 2001. These decreases were clearly due not only to the development of 3Rs (i.e., Reduction, Refinement, and Replacement of animal use) in alternative research, but also to marked changes in the focus of experimental animal biology. In the academia, animal experiments using wild-type mice have decreased in number to a large extent relative to those using genetically modified mice because of the mechanistically much reliable outcomes obtained by genetically modified mice than those from wild-type animals. Yet, biological safety studies for pharmaceutical development as well as industrial chemical safety studies utilize conventional toxicological bioassays.

Keywords: 3Rs, Claude Bernard, Bruce N. Ames, Patric O. Brown

Introduction

Historically, three scientists are recognized in relation to the history of experimental animal use; the first, the initiator of experimental animal research; the second, the first contributor to the marked reduction of the number of experimental animals used; and the third, a potential contributor, who invented an ultimate method for reducing the number of animals for future research, the gene chip technology. The use of animals in experimental studies was initiated by Claude Bernard (1813-1878), originally who was trying to put an end to human vivisections common at that time; thus, he came to be regarded as "the devil of experimental animals." The most remarkable contribution to reducing the number of experimental animals used was made by Bruce N. Ames, who rescued innumerable animals that might have been used for genotoxic carcinogenicity studies. Another contribution may be attributed to Patrick O. Brown, who invented transcriptomics, which can be used to elucidate the underlying mechanistic background of phenotypes of experimental animals; the method is considered to have eventually led to the minimization of experimental animal use. Consequently, the most essential and powerful driving force for future alternatives may be minding the 3Rs but also the promotion of basic sciences and technologies.

1. Claude Bernard – An initiator of animal experiments

Claude Bernard (1813-1878)

Bernard was born in the village of Sain-Julien in 1813, and went to Paris at the age of twenty-one. As reported, he first wanted to be a play wright, but took up medical studies on the advice of a literary person. He learned medical science from the famous Françoise Magendie, and earned his PhD after pursuing the study of gastric acids. He was appointed as Magendie's deputy professor at the college in 1847, and made seminal discoveries such as those of hepatic glycogen, vasomotor neurons, and curare

narcosis. Any of these discoveries must have made him an accomplished medical scientist. Because of his scientific principles, he strictly defined *observers* and *experimenters*, critically. He called observers as those who do not alter "nature", but statically observe the ostensible world; whereas experimenters are those who purposely alter "nature" to obtain a reaction, and seek natural responses behind the phenomenal world. He strongly recommended the use of living organisms to obtain responses, and seek natural reactions behind the phenomenal world. This is the reason why he emphasized the use of vivisection in science throughout his life. In his major discourse on scientific methods, "An Introduction to the Study of Experimental Medicine" (1865), Claude Bernard described what makes a scientific theory good and what makes a scientist important and a true discoverer. Unlike many scientific writers of his time, Bernard writes about his own experiments and thoughts, and uses the first person[2].

Although Bernard was the first scientist who initiated the use of animals in experiments, his original aims at that time were to criticize physicians and to rescue humans from iatrogenic accidents due to poor and insufficient surgical treatments. However, his wife and daughter initiated the first "animal rights campaign" immediately after his death[3], because of their intense aversion to Bernard's animal studies without using anesthesia, namely, vivisection, although this is ironically the best and appropriate method of determining the response of experimental animals.

It is about a century since Bernard started a systematic education on animal experiments. Experimental studies using animals changed last decades because of not only a greater awareness about animal welfare, but also greater decreases in the need for conventional experiments. Accordingly, in 1984, the International Guiding Principles for Biomedical Research Involving Animals was established. Then, in 1985, the European Convention also established the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes.

In Japan as well also, laws for animal care were successively passed in the 1970's. The Act for Animal Welfare and Proper Administration was passed in 1973, and the Guidance Documents for Experimental Animal Maintenance and Proper Administration in 1980. The Guideline for Experimental Animal Use was established in the same year in 1980 by the Japanese Academy of Science, the Guideline for Proper Use of Experimental Animals in 1987 by the College Union, and the Extension of Animal Life and Ethics by the Japanese Academy of Science in 1996. Recently, the establishment of the Act for Animal Welfare and Proper Use in Experiment was issued in 1999. Despite these guidelines, we could not eliminate all the animal experiments at the moment. However, we are now at the turning point in the history of experimental animal use.

2. Bruce N. Ames – accomplished the most prominent alternative study –

Bruce N. Ames

We now introduce a scientist who developed the revertant mutagenesis assay, Bruce Ames[4]. Ames is now a Professor of Biochemistry and Molecular Biology at the University of California, Berkeley. He is a member of the National Academy of Sciences and he was on their commission on life sciences. His publications of more than 450 led to his being among the most cited scientists.

The idea of mutation induced by chemical compounds was first described in 1944[5-6]; this was about 30 years prior to Ames' development of revertant mutagenicity assay. Chemical mutagenesis became the focus of considerable attention, because large amounts of industrial chemicals started to be used in various industries in the mid-twentieth century. Afterwards, because Ames' test enabled the detection of most mutagenic compounds, it has contributed greatly to a large reduction of the number of experimental animals used for in vivo mutagenicity bioassays. What Bruce Ames originally proposed was to use an induced bacterial gene mutation as an evaluation tool for mammalian mutagenesis. He attempted to develop a system for incorporating mammalian microsomal metabolism to the assay also by him, which is presently known as the S9-mixture[7]. It took a very long-time to establish the test system after considerable debate, because scientists at that time had to learn the difference between direct genotoxic carcinogenesis and indirect genotoxic carcinogenesis, namely, epigenetic carcinogenesis. However, after the establishment of the test system, innumerable experimental rats and mice were saved from carcinogenicity bioassay. Although Ames did not intend to save experimental animals by his invention, knowing such common rule of mutagenicity in genetics between Salmonella and mammalians made innumerable number of reduction in experimental animal use possible. Thus, from the study of Ames, our conclusion on alternative studies, is, that an

essential strategy for reaching an alternative goal may be the "Development of True Sciences."

The current major interests of Bruce N. Ames are reported to be the determination of optimum micronutrient intake for minimizing human DNA damage as a preventive measure against cancer, and the study of other degenerative diseases associated with aging.[8-9]

3. Patric O. Brown – gene chip technology

Patrick O. Brown

The third person who we introduced is Patrick Brown, who invented a new methodology, that is, gene chip technology[10]. The gene chip technology and the consequent toxicogenomics[11] that he developed were supposed to rapidly minimize experimental animal use to a large extent[12] (Meeting proceedings from ECVAM-ICCVAM/NICEATM, 2006).

The establishment of the genome sequencing program in 2000 was supposed to be a strong driving force for the progress of alternative studies, particularly via toxicogenomics. All the information derived from animal experiments is incorporated in the genome expression database, that is, "computer mouse", which may be virtually used in the near future even without actual animal experiments.

The method established by Patrick Brown is "molecular microscopy", which enables the differentiation of patterns of gene expression profiles[13]. We showed sample expression profiles of genotoxic compounds studied by the consortium of International Life Science Institute (ILSI), which showed a short-term differential prediction of chemicals with DNA-binding affinity, such as cisplatin, methotrexate, mitomycin C, and chemicals with indirect genotoxicity, such as, taxol, hydroxiurea, and etoposide. Such a rapid and easy prediction may greatly contribute to the realization of essential purposes leading to the development of 3Rs.

Concerning the gene expression profiles, linear increase in dose-response relationship obtained by a conventional testing protocol may not be always applicable each other. In the presented example of microarray data after radiation exposure, because the expression levels of some genes increase with radiation dose and those of some genes decrease with increasing radiation dose, the dose-response relationship obtained by a conventional toxicological testing protocol can be assumed as the only phenomenologic outcome on the basis of one aspect. Rather, we recommend that the dose-response relationship should be considered complex, and that these combination profiles per se, may be essential biomarkers. The authors showed other sample data obtained after whole-body radiation in which one can observe dose-related expression profiles, on one hand, and dose-specific expression profilings, on the other.

Another issue that the authors introduced was age-related stochastic and probabilistic gene expression profilings, which can also be visualized in nontreated senescent mice when one focuses on their individual gene expression. By linear configuration for gene expression, one can clearly recognize that the divergent expression profiles of each individual mouse were not due to an error, but biological diversity with aging. Moreover, representative responsible genes showed clear differences between 2-month- and 21-month-old profiles, which elucidated the age-related responsible gene ontology, represented by the senescence-specific genes[14].

In the cases of experimental myeloid leukemias, spontaneous leukemias are differentiated from those of radiation-induced myeloid leukemias by their different responsible gene intensities in the line configuration of the expression gene profilings. They are also differentiated by the analysis of principal components, which are observed from the three dimensional expression. These databases are also supposed to be essential information for developing 3Rs supported by basic science.

Toxicogenomics sometimes makes the categorical border between physiology and toxicology ambiguous. Similar genes, such as those encoding apoptosis-related genes, caspases, participate simultaneously as physiologic and toxicologic parameters. Toxicogenomics sometimes changes a toxicologic paradigm. Depending on such fluctuating changes in the cell cycle genes, for example, and many other cellular functions, which may be mild or severe, the degree of oscillatory ranges differs from one another, which may be new risk factors.

Conclusion

Lastly, as we mentioned above, the use of experimental animals has, unfortunately, not been completely eliminated to date. Thus, in this regard, we would like to emphasize that "science should progress further". Certainly, one may not accept any risky drugs that have not undergone preclinical animal testing for use in one's children. On the other hand, no one may believe that animal studies will be continued for more than 4-500 years from now. We believe that experimental animals may be eventually replaced by other technical systems developed in the future, although such systems are still technologically immature to replace everything at this moment.

Table 1. Surveillance of experimental animals used in Japan.

	1995	1998	2001
rats*	2.09 (100)	1.53 (73)	1.24 (59)
mice *	6.68 (100)	[redacted]	2.80 (42)

* Million / (%)

Surveyed by the Society of Experimental Animals in Japan[1].

The authors emphasized that animal testing may be eventually replaced by other new technologies, and animal testing would eventually disappear. Some people, however, believe that animal testing should be replaced immediately by other technologies; hopefully today, if not today, maybe tomorrow! These gaps may be filled by nominal driving forces such as humane animal welfare, industrial economy, and politics. However, the essential driving force for this matter may be the development of science itself, particularly by the development of "genome sciences". In other words, an elimination of animal experiments may be l'oiseau bleu (blue bird) of each scientist for the development of future science.

A recent survey by the Experimental Animal Society of Japan showed marked decreases in the number of experimental animals used[15]. As shown in the **Table 1**, the numbers of rats and mice used decreased to 59% and 42%, respectively, since 1995. The possible reason for these decreases is the obtainment of considerably clear-cut experimental results using a relatively small number of genetically modified animals, whereas unreliable experimental results are obtained with a relatively larger number of wild-type animals. These data strongly suggest the future possible reduction in the use of experimental animals.

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Biophilia special

内分泌かく乱化学物質研究の世界的動向

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1996年にロンドン郊外のWeybridgeで、内分泌かく乱化学物質に関する初めての国際ワークショップが開催されてから10年が過ぎ、昨2007年1年間は、さまざまな回顧と展望が語られた(2007年5月は、DDTの全面禁止につながる『沈黙の春』の著者レイチェル・カーソンの生誕100年でもあった)。この間、2002年には、WHOがグローバルアセスメントをまとめ、2005年11月には、Weybridge10周年を記念したワークショップがヘルシンキで開催され、研究の進捗状況が報告された。

本稿では、内分泌かく乱化学物質研究を、ヒトを含む野生生物が環境との間に営む生体異物相互作用と捉え、この10年間の研究のあゆみの中から、今後の研究に求められていることを述べてみたい。

Weybridgeワークショップからヘルシンキ会議まで

当時、ワニ、カメ、あるいはカモメなど種々の野生生物では、それぞれ血漿エストラジオールの減少、ヴィテロジェニン(卵黄タンパク成分)の増加、あるいは卵殻の菲薄化等、さまざまな異常生態が観察され、それがジコフォール、DDT、あるいはPCBsなどさまざまな農薬、化学物質によるものではないかと危惧され、話題になりはじめていた。これはさらに、ヒトで、尿道下裂のような小児の先天性奇形、あるいは俗にキレるといった表現で表される小児の精神神経学的な障害、さらに乳がんや前立腺がんなどの頻度の亢進などにも、関連があるものとされるに至った(図1)。

こうした中で開かれた欧州連合の主催によるWeybridge会議では、世界保健機構(WHO)と経済開発協力機構(OECD)がそれぞれ役割を決め、この問題に協力

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横浜市立大学医学部卒。東京都老人総合研究所、米国ブルックヘブン国立研究所、放射線医学総合研究所などを経て、2001年より現職。専門は、実験病理学、実験動物学、分子毒性学。現在は化学物質の生体影響発現機構の研究を進めている。著書に、「Toxicogenomics」(編著、Springer-Verlag社、2003年)など。

危惧された表徴所見	生物学的蓋然性	疫学的所見	研究課題
先天性奇形（尿道下裂など）	yes	???	発生障害の機構研究（含：胎生期ウィンドウ問題、核内受容体問題など）
生殖能の低下	yes	???	繁殖毒性障害の機構研究 化学物質とホルモン受容体の相互作用研究
精神神経学的障害	yes	???	高次生命系のかく乱の可能性（中枢神経系、内分泌系、免疫系の発生生物学）
発がん性の亢進（乳がん・前立腺がん）	????	???	組織特異的遺伝子発現;エストロゲン・シグナル応答性遺伝子発現
野生生物所見（雌雄差、など）	yes & ???	——	感受性の種差を裏付ける機構研究（種差の特徴; 生殖腺の形態形成）
低用量反応	yes	——	受容体原性毒性反応機構
相乗／相加反応	yes	——	エストロジェンシグナル応答性遺伝子発現

（米国学士院）

図1　内分泌かく乱仮説と生物学的蓋然性

して取り組むことになった。その申し合わせの1つとして、WHOは内分泌かく乱問題に関する世界的なアセスメント、グローバルアセスメントの編纂を20人ほどの編集委員をもって、3年計画で開始した。編集委員会では個々の事象を1つ1つ検討し、2002年、それまでその存在の如何についてもはっきりしていなかった内分泌かく乱現象に対し、最終的に、内分泌かく乱物質問題がすでに存在する概知の事象であろうと結論づけ、出版物として刊行した。

この時のまとめは4点あり、1)身体が形成されていく過程での曝露が、刺激に対する応答機能を恒久的に変化させてしまう可能性をもつこと、2)成熟した動物への曝露は、ホメオスタシス(恒常性)に基づいた応答により、顕著な影響を示さないかもしれないこと、3)発育段階の違いや季節変動などで同じホルモン様の影響が異なった結果を呈する可能性のあること、そして、4)内分泌系の異なった要素間でのクロストークにより予想外の影響を生ずる可能性のあることなどを挙げた。そして何より、内分泌かく乱化学物質問題は仮説の問題ではなく、すでに存在する既知のことがらであること、従って何らかの対応が必要なことを結論したことがその後に与えた影響には顕著なものがある。

グローバルアセスメントでは、内分泌かく乱化学物質が、成獣には影響を起こしにくいようであること、しかし、胎生期と新生児期の性成熟過程にあたる形態形成期では不可逆的影響を及ぼす可能性がある、といった点を強調した。この指摘により欧州機構や各国の取り組みが本格化した。昨年末のヘルシンキ会議は、さらに5年経って欧州機構などのこの問題に関する研究支援が本格的に軌道に乗った中で行われ、多くの新しい有用な研究情報が紹介された。会議最終日、ダイオキシン受容体研究で知られるジューコ・ツオミストが挨拶に立ち、野生生物の深刻な実態に比較した時のヒトのリスクを次のようにまとめた。「多くの内分泌かく乱物質は、経口的に摂

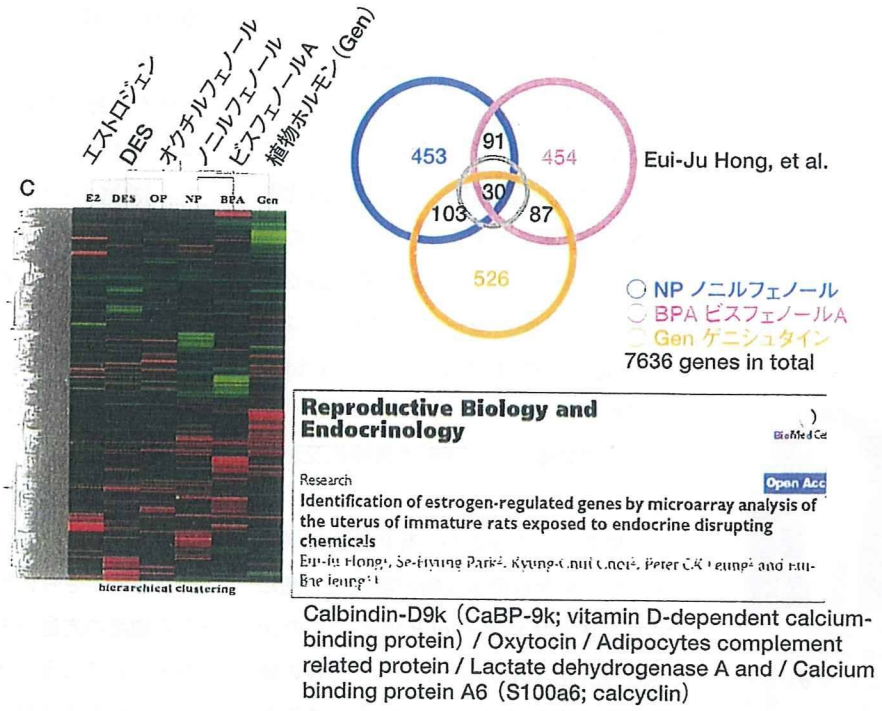

図2　遺伝子発現で見た内分泌かく乱化学物質の性質

左のキルト模様のようなグラフは、生体がどう反応しているのかを遺伝子の反応パターンで示している。左図のエストロジェン、DES、オクチルフェノールなどは、いずれも女性ホルモン様の作用をもつとされている。しかし、グラフに明らかなとおり、横一直線に共通した反応は見られない。右のバイグラフも同様であり、ノニルフェノール、ビスフェノールA、ゲニシュタインの3つの化学物質で共通に発現する遺伝子は30個検出されるのみである。つまり、内分泌かく乱化学物質といっても、ヒトに共通に影響を与える目印になる遺伝子は見られず、したがって、十把一絡げにその特徴を判断するわけには行かない、ということがわかる。

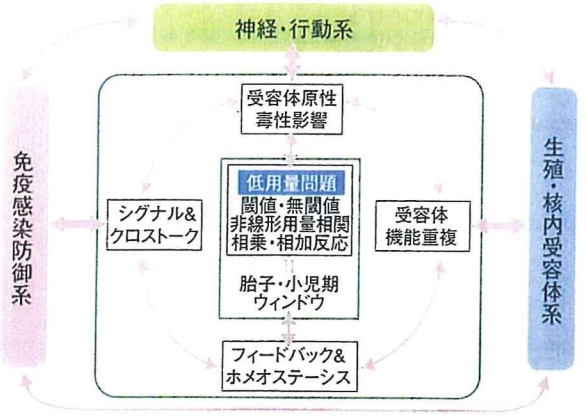

図3　分泌かく乱化学物質の高次生命系への影響（模式図）
神経・行動系、免疫感染防御系、そして生殖・核内受容体系、の3系統に重点を置いた研究が進められている。これらの臓器では、ホルモン受容体が普段から発現していて、共通のさまざまな因子の発現が観察される。それぞれが長期の記憶装置をもっていることも特徴である。

取されるであろう。しかし、ヒトの摂取する食物と水とは、その衛生管理が整っており、そこで、ヒトへの曝露は、事実上認められない」というものである。おそらく、そうした紙一重の状態が、ヒトへのリスクを押さえているというのが実態なのだろう。

新しくわかったこと、今後必要性な研究

以上がこれまでの概略であるが、初期、危惧されていた事柄などを通覧すると、人々の身のまわりにはホルモン様物質がたくさんあり、その中では生体ホルモンでさえ、本来、ある程度生体に有害な性質をもつ。雌雄対偶動物の身体の中ではこれが"漏れ出さないよう"緻密な自己防護システムが備わっていること、あるいは内分泌かく乱化学物質とよばれるものに、物質としての共通作用の乏しいこと（図2）、さらには、そうした化学物質の複合作用の有無など、多くの事柄で、多分に整理されないまま、机上の論議が行われてきた感が否めず、実験生物学の立場から見ると、充分に的を射た議論が行われてこなかったように思われる。

他方、筆者らは、内分泌かく乱化学物質の生体影響研究では、ホルモン受容体が普段から発現していて、いろいろな共通の補助因子の発現が観察され、それぞれが長期の記憶装置をもっていること、神経・行動系、免疫・感染防御系、そして生殖・核内受容体系などの諸系統に注目することの重要性を、これまでにも啓蒙書[1]を出版したりして強調してきた（図3）。幸いなことに、この考えはかなり妥当な判断だったようで、一連の研究からは、0.01～0.2mg／kg／dayという無作用量以下の低用量のビスフェノールAに、神経・行動異常を引き起こす作用が見出されており[2]、諸分野で、注目すべき新たな知見と今後の研究の展望が明らかに成りつつある。ここでは、特記すべき点のみ、かいつまんで紹介する。

1．用量－反応曲線と低用量への外挿性

毒性学による安全性の試験では、高用量の反応から直線回帰をして低い用量での反応性を予測することが多い。したがって、低用量での作用が明らかになりつつある内分泌かく乱化学物質の作用曲線について、無作用量以下の低用量域で外挿表徴型と異なった反応がないかどうか

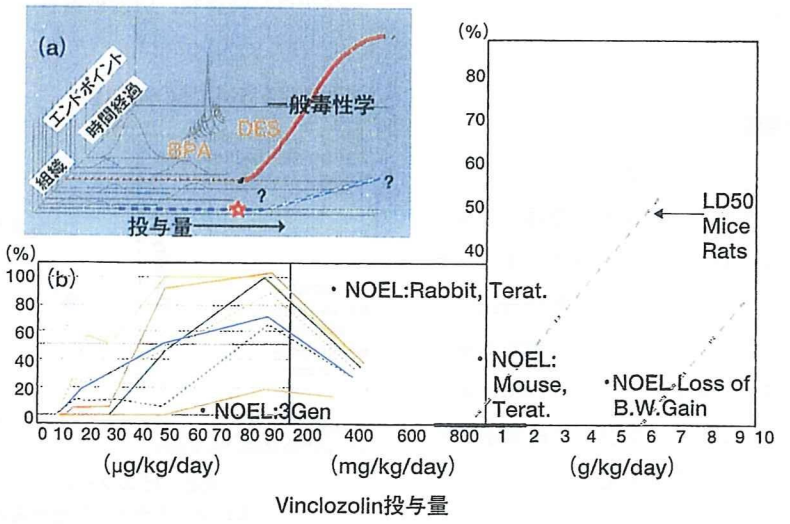

図4　低用量域の変化と稀少特性
既存のデータを整理すると、従来は、無作用量、無毒性量と定めていた用量より低い濃度で、さまざまな影響が観察されることが報告されている。
（b：米国の国立環境影響研究所NIEERLのEarl Gray博士による）

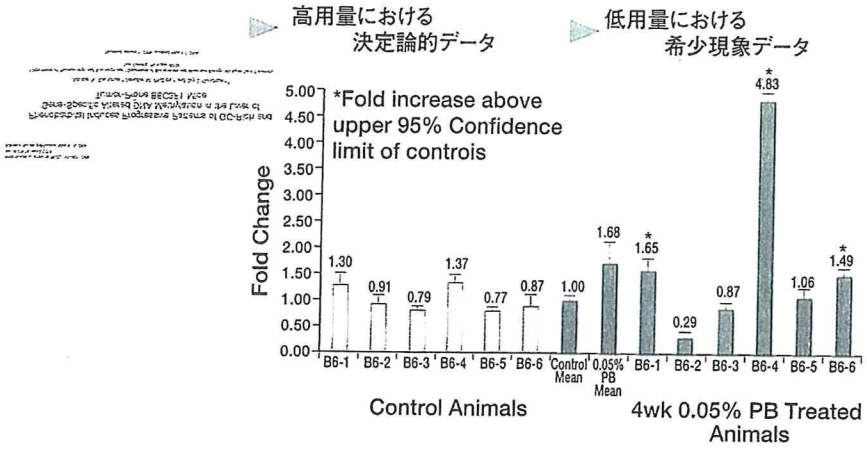

図5　低用量域の変化と稀少特性

低用量域で種々の試験法に沿って実験をすると、純系動物を用いた実験でも、変動幅の広い結果得られることがある。図は、フェノバルビタールによるメチル化部位の形成確率を、ネズミ一匹毎に検出したもの。対照群と異なり、個体毎の値が広く分布している。内分泌かく乱化学物質影響にもこうしたエピジェネティック特性が想定される。(ミシガン州立大学のJay Goodman教授による)

の如何については、早くから議論があった。そして実際に既存のデータを整理すると、従来無作用量とか、無毒性量と定めた用量より低い濃度でさまざまなデータが認められた。米国環境防護庁所轄の国立環境影響研究センターでは多年度計画を立てて検討に入っている[3] (図4)。

2. 低用量反応の問題点

メチル化は、たとえ純系動物でも確率論的に形成され、等質の結果が出ないことがわかってきた (図5)。こうした結果に対して平均値を取ると、ばらつきは背景データに隠れてしまうので、“プロクラステスのベッド”*で知られる通り、禁忌とされる。

*プロクラテスは、一面に宝石を散りばめた黄金のベッドの寸法よりも客の寸法が短いと、ベッドの寸法に合うよう4人の力持ちの大男に引き延ばさせたり、ベッドよりも長いと頭や足を切り落とさせたという。

3. 発がん蓋然性の問題

発がん性との関係では、発がんの蓋然性、つまり、これらの物質によってがんが起こりやすそうな体内環境が形成される可能性があるのかどうかという点である。女性ホルモンとダイオキシン類の1つが、正常のmycという遺伝子と協同して、テロメラーゼという、細胞を無限増殖へ導く遺伝子の活性を引き上げた、という報告がある[4]。こうした変化は、がん化につながる可能性があるため、早急に検証することが必要である。

4. 思春期早発の蓋然性と加齢影響

内分泌かく乱化学物質の性質の1つとして、思春期を早く発来させ、早期の老化を引き起こすなどといった点に危惧があった。図6に示したように、ビスフェノールAの効果では、投与した動物の寿命曲線が、グラフの白○印の対象に較べて、死亡が早期化し、傾きも急峻になる傾向があることがわかる[5]。もしこの寿命曲線が正しいとすると、これまで見てきた生殖リスク評価センター (CERHR) の判断とはまったく違った結果になるわけであり、これについても、よく調べる必要がある。

5. 内分泌器官の拡張

さて、次に注目すべき点は、結論から述べると、これまで内分泌器官と一括して述べたが、よく調べてみると、肝臓や脂肪細胞など、従来必ずしも内分泌器官と考えてこなかった臓器が、内分泌器官の役割をしていると、い

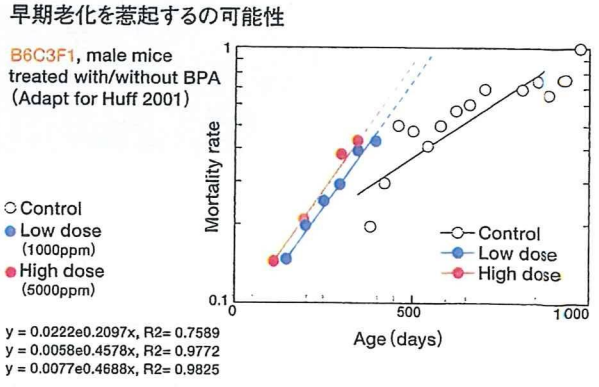

図6　ビスフェノールAによる
エピジェネティック発がんと促進老化

内分泌かく乱化学物質の性質の1つとして、思春期の早発や早期老化が危惧されてきた。ビスフェノールAを投与した動物の寿命曲線は、白○印の対象に較べて、死亡が早期化し、傾きも急峻になる傾向が見られる。ビスフェノールAは、エピジェネティック発がんの促進が見られるようである。(文献5より引用改変)