Tab. 24.3 Major changes in gene expression profiles in wild-type (WT) and p53-knockout (KO) mice. Mice exposed to 300 ppm benzene for 6 h d^{-1}, 5 d wk^{-1}, for almost 2 weeks and killed on day 12.[a]

Gene name or name of protein encoded by the gene	*Fold change*		*Accession number*
	WT	*KO*	
Aldehyde dehydrogenase 4	1.07	2.44	U14390
Apoptotic protease activating factor 1 (Apaf-1)	1.16	1.75	AF064071
Bax-alpha	1.20	1.21	L22472
Bcl-2 alpha	0.91	1.66	L31532
Calcyclin	1.08	1.89	X66449
Caspase-9	0.83	1.59	AB019600
Caspase-9S	0.84	2.26	AB019601
Caspase-11	2.49	1.22	Y13089
Caspase-12	0.86	0.18	Y13090
c-fos	1.57	0.94	V00727
Cyclin B1	0.85	1.48	X64713
Cyclin D1[b]	0.44	(–)	M64403
Cyclin D3	0.83	1.20	M86186
cyclin G1	1.67	1.32	L49507
CYP2E1	2.13	1.72	X01026
Cyclin D-interacting myb-like protein (Dmp1)	2.01	2.81	U70017
Elongation factor 1[b]	3.12	(–)	AF304351
Gadd 45[b]	1.63	(–)	U00937
Glyceraldehyde-3-phosphate dehydrogenase	1.06	3.34	M32599
G protein-coupled receptor (GPCR/EB11)	0.01	0.97	L31580
JNK2	1.07	1.82	AB005664
KSR1, protein kinase related to Raf protein kinase	1.11	2.57	U43585
Lactate dehydrogenase 1 (LDH1)	1.13	2.34	AW123952
Lactate dehydrogenase 2 (LDH2)	0.97	1.72	X51905
Metallothionein 1	4.89	0.93	V00835
Metaxin2	0.95	1.55	AF053550
mLimk1, *Mus musculus* protein kinase	2.67	1.18	X86569
Mph1/Rae 28, polycomb binding protein	4.97	0.06	U63386
Myeloperoxidase (MPO)	1.68	1.49	X15378
p21[b]	1.37	(–)	U09507
p53, variant mRNA	1.03	0.13	U59758
p58, protein kinase inhibitor (PKI)	1.55	0.81	U28423
PERK, ER resident kinase	0.81	1.63	AF076681
PI3K catalytic subunit p110 delta	2.36	0.18	U86587
RAB17, member of RAS oncogene family	2.42	1.53	X70804
Rad50	1.23	0.40	U66887
Rad51	0.72	0.08	AV311591
Rad54[b]	1.50	(–)	AV311591
Siva, pro-apoptotic protein	0.88	1.62	AF033115
Smad6	1.36	1.92	AF010133
Serum inducible kinase (SNK)	1.68	1.02	M96163
Superoxide dismutase, Cu/Zn	1.19	1.63	M35725
Topoisomerase III[b]	1.90	(–)	AB006074
Tuberous sclerosis 2 (Tsc-2)	2.00	1.25	U37775

Tab. 24.3 (continued)

Gene name or name of protein encoded by the gene	Fold change WT	KO	Accession number
Wee-1 [b]	1.95	(–)	D30743
Wig-1, p53-inducible zinc finger protein	1.83	0.07	AF012923
WISP1	0.68	1.26	AF100777
WISP2	0.83	8.32	AF100778
Wnt-1/INT-1	1.72	1.23	M11943

a) The studies involved two to four animals and the data were obtained by using Affymetrix gene chips. Mice were killed on day 12 immediately after benzene exposure.
b) No data available for p53 KO mice.

major selected genes whose expression showed p53-dependent benzene-induced decrease or increase (gene for G protein-coupled receptor 1), or in which gene expression was abolished in p53 KO mice. Cyclin genes, such as *cyclin B1* and *cyclin D3*, were generally activated in p53 KO mice by benzene exposure; in contrast, cell-cycle suppressor genes, including the G2–M cycle checkpoint gene, *p58* [68], were up-regulated in WT mice. These findings are compatible with the idea that the hematopoietic cell cycle continues in p53 KO mice even during benzene exposure, whereas it is arrested due to alterations in the expression of cell-cycle checkpoint genes, particularly the p53 gene, in WT mice. Such information may be very important for understanding yet-unknown toxicity mechanisms of chemicals. It is important to note here that such conclusions may be drawn by carefully and simultaneously screening different expression patterns of many genes having interrelated functions, even genes that show only small changes in expression level (about 1.5 to 2 fold). Investigation of the expression of a limited number of genes generally may not provide an insight into the main mechanism of action of chemicals or clues about the particular role of each of the investigated genes involved in the mechanism. Toxicogenomics may have a strong advantage from that point of view, as is also well described in the literature [7].

Ivanova et al. listed 17 genes including three EST genes in the cell cycle regulators among the genes profiled as stemness indicators [35]. Among them, 12 genes were confirmed in the list of genes expressed in the steady-state bone marrow of our present study. The expression levels of these 12 genes were all nearly comparable to that of beta actins; that is, comparable to the percent of the 'stem cell' concentration.

Two cell cycle regulator genes, *Wee 1* (D30743; one of the 17 mentioned above [35]) and *Mph1/Rae28/Edr1* (U63386; a member of polycomb, classified as one of the chromatin regulator genes in the stemness profile [35]), were significantly expressed after benzene exposure and were identified as possible candidates of marker genes for benzene exposure. Whether these possible marker genes for benzene exposure represent a change in an expression profile of stemness or are genes expressed in new, reacting progenitor cells after benzene exposure is not known; however, a possible role of these two genes as marker genes for benzene exposure is of much interest.

24.5.4
Apoptosis-related Genes in p53 KO and WT Mice

The microarray analysis results of p53 KO mice reminded us of the important role of the p53 gene in the mechanism of action of benzene. The genes regulated by the p53 gene, including *p21* [64], *caspase 11* [69], *PIK3K* [70] and *cyclin G1* [71], were distinctly up-regulated in the benzene-exposed WT mice (Table 24.3). It is of great interest that *caspase 11* rather than *caspase 9* was highly expressed after benzene exposure, suggesting that the p53-mediated activation of *caspase 11* is an important signalling pathway for apoptosis of bone marrow cells triggered by benzene exposure. This novel observation associated with the benzene toxicity mechanism, together with down-modulation of *caspase 12*, was similarly addressed in a study of the mechanism of chronic obstructive urinary disturbances, in which p53 KO and p53 WT mice were used [69]. On the other hand, the up-regulation and down-modulation of genes associated with oxidative stress was shown in p53 KO mice, which suggests that benzene may have produced oxidative stress in these mice (Table 24.3) [65]. It is not clear why oxidative-stress–associated genes are activated in p53 KO mice but not in WT mice; this may reflect deregulation of the redox cycle due to the absence of the p53 gene and the consecutive counteractivation of antioxidant enzymes [72]. The genes encoding apoptotic protease activating factor 1 (*Apaf-1*) and metaxin and the *Siva* gene were also up-regulated in the benzene-exposed p53 KO mice [65]. The expression of these genes may suggest that pro-apoptotic conditions are induced by benzene exposure of p53 KO mice. It was, however, found that survival or anti-apoptosis genes such as *bcl-2*, *caspase 9S* (an endogenous dominant-negative form of *caspase-9* [73]), and *Smad6* (an antagonist of the TGF-beta signalling [74]) gene, were also activated in p53-KO mice (Table 24.3) [65]. The up-regulation of the gene for PERK (ER transmembrane protein kinase) in p53 KO mice [65] indicates a triggering of the unfolded protein response (UPR) signalling pathway, resulting in a loss of *cyclin D1* [75] (which was statistically less confident in the present data).

24.5.5
DNA-repair–related Genes in the p53 Gene Network

Despite the possible DNA damage in bone marrow cells from p53 KO mice, the DNA repair system is not likely to be functioning efficiently in these mice, since DNA repair genes that were actively functioning in the WT mice exposed to benzene were not activated but rather suppressed in the p53 KO mice [65]. In association with cell proliferation and apoptosis, high expression levels of the *tuberous sclerosis* gene (*Tsc-2*), a tumour-suppressor gene encoding tuberin, and the gene encoding metallothionein 1 were noted in the WT mice (Table 24.3), raising the possibility that these genes are regulated by p53. The association of metallothionein with p53 transcriptional activity was recently postulated after study of an *in vitro* system in which metallothionein acts as a potent chelator to remove zinc from p53, thereby modulating p53 transcriptional activity [76]. The *Tsc-2* gene was recently reported to regulate the insulin signalling pathway mediated by AKT/PKB for cell growth [77, 78]. It is

noteworthy that *Tsc-2* is a target gene of 2,3,5-tris(glutathione-*S*-y) hydroquinone, a metabolite of hydroquinone for renal cell transformation [79]. The high expression level of the *mph1* (rae28) gene in the WT mice (Table 24.3) [65] along with severe depression of bone marrow cells was interesting in association with the sustained activity of hematopoietic stem cells [80]. Furthermore, the *Wnt-1* signalling pathway was also likely to be activated by benzene exposure. Aberrant expression of downstream genes such as *WISP1* and *WISP2* did not occur in the WT mice, but such expression was evident in the p53 KO [65]. Since the Wnt-1 signalling pathway is reported to regulate the proliferation and survival of various types of stem cells, including B-lymphocytes [81], the activation of both the *mph1* and *Wnt-1* genes may be associated with the rapid recovery of suppressed bone marrow cellularity after termination of benzene exposure. Some upstream genes encoding p53, such as those encoding cyclin D-interacting myb-like protein (*Dmp1*) and *KSR1* (protein kinase related to *Raf*) in the p53 KO mice, compared with those of the corresponding experimental group of WT mice, were up-regulated to a similar extent or even strongly enhanced in their expression (Table 24.3) [65]. This is another indication of the role of the p53-mediated pathway in the mechanism of action of benzene associated with cell cycle regulation.

Finally, by analyzing gene expression profiles, one can elucidate the mechanisms underlying benzene hematotoxicity. The next step is to further analyze the fractionated stem cell compartment and compare the results with those reported by Ivanova et al. [35]. For example, *CYP2E1* is known to be constitutively expressed in the WT mice [62], which was confirmed in the present investigation (Table 24.3). Interestingly, *CYP2E1* gene expression is in the list of Ivanova et al. [35]. Since the list was established by subtraction of the expression of cells other than stem cells from the expression in stem cell compartment, the expression of the *CYP2E1* gene targeted by benzene toxicity is assumed to be an exact reflection of benzene-induced stemness toxicity.

24.6 Summary

Molecular biology has enabled the elucidation of biological subjects by two strategies, namely, inductive and deductive approaches. The progress in the mouse whole-genome sequencing project has enabled the elucidation of bilateral interrelationships between toxicological phenotypes related to particular toxicants and expression profiles of pertinent genes induced by exposure to toxicants [7]. Since all the phenotypes observed through various traditional toxicological tests should be eventually linked to gene expression profiles, translation between phenotypes and gene expression profiles provides a promising tool for predictive toxicology, despite some phenotypes being expressed due to various nongenomic signal transductions. In fact, many gene expression trials have been performed to provide adequate molecular biological information on the underlying mechanism of such phenotypes. In this chapter, hematotoxic gene expression profiles after a single dose of 300 cGy irradiation or

repeated inhalation of 300 ppm benzene for 6 h a day, 5 days a week, for 2 weeks were introduced as an inductive approach of toxicogenomics. Then, a couple of plausible genes were selected with respect to the 'stemness profile' and discussed as a deductive approach for possible hematotoxicological applications of toxicogenomics. We did not incorporate data on clinical diagnosis, responsiveness to treatment, nor prognosis in this chapter, but considerable predictability has been shown in such trials elsewhere [83, 84].

Two major unresolved questions that we may have to pay specific attention to at this time are how one can define a specific mechanistic interpretation of the expression profiles for each discontinuous independent parameter; and how one can define a possible predictability of gene expression profiles (for carcinogenicity, for example) not only for the chemical group for which data were compiled during establishment of a database, but also for a group for which data were not incorporated into the database, that is, unknown chemicals. These unknown subjects should be given focus in future trials in toxicogenomics.

Finally, we have to note that the application of toxicogenomics to hematotoxicology should eventually focus on changes in the expression profiles of the hematopoietic stem cell/progenitor compartment, although the presented case study did not fully focus on this issue. In the WT mice, up-regulation of the *p53* gene did not appear in two weeks after intermittent benzene exposure, but was weakly detected a month after 300 cGy irradiation. Up-regulation of *cyclin G1*, downstream of *p53*, was observed after both benzene inhalation and 300 cGy irradiation, implying that the up-regulation of *cyclin G1* may be a relevant reflection of prolonged DNA damage.

When benzene was administered, down-regulation of *caspase 12* and up-regulation of *cyclin B1* was seen only when *p53* was knocked out (these gene changes may be hidden by *p53* gene regulation in WT mice) [65]. Participation of the same gene repertoire as in benzene exposure is observed even in the WT mice one month after 300-cGy irradiation, suggesting that the result may reflect a possible *p53* dysfunction in these irradiated mice.

For the future, more precise comparison between common gene expressions in the expression profiles in hematopoietic stem/progenitor cells after radiation exposure and benzene inhalation and the expression of genes on the stemness list compiled by Ivanova et al. [35] may lead to a clearer understanding of a possible marker repertoire of stem/progenitor cells for general hematotoxicological responses.

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POTENTIAL APPLICATIONS OF TOXICOGENOMICS IN RISK ASSESSMENT

Tohru Inoue

Center for Biological Safety and Research, National Institute of Health Sciences, 1-18-1 Kamiyohga, Setagayaku, Tokyo 158-8501, JAPAN

The concept of toxicological risk identification using toxicogenomics is not well characterized yet, because of its different paradigm from the conventional toxicologic testing scheme. Toxicogenomics is based on a high-density microarray for mRNA expression which reflects and includes whole genomic function from physiological activities to toxicological alterations, such as in acceleration and deceleration of cell cycling, in cellular proliferation and apoptosis, or in self-renewal and differentiation, and various metabolic activities. Thus, genomic functions from physiological activities to toxicological alterations form a "continuum". A risk border can exist somewhere in the continuum between the physiological and toxicological oscillations of gene functions. In the presentation, because of such underlying new paradigm of toxicogenomics, the concept of the methodology of toxicogenomics is overviewed, and then the toxicological application as a risk assessment will be discussed.

Toxicology is an interdisciplinary applied science that is between "applied biology", which includes veterinary and human medicine, and "applied chemistry and physics". Various unclarified factors of methodological steps in toxicology have been recognized in the former, that is, biology and medicine, which seem to be due, in part, to unknown factors represented by a difficulty in interspecies extrapolation, inter- and intraspecies differences based on species and/or individual genetic differences (e.g., single nucleotide polymorphisms (SNPs)), and nonlinear complexity in unclarified responses to xenobiotics based on unknown gene regulations including uncountable biological signaling networks. Such unknown black-boxed factors are going to be disclosed by a newly developing field of "toxicogenomics", "transcriptomics". Toxicology and the identification of pharmacologic toxicity are different: The former should define not only possible toxicities, but also "nothing", that is, to define a "nontoxic border"; whereas, the latter, is related to the identification of "something targeted endpoints", either at the pharmacological toxicity level or pharmacological side effects, in addition to pharmacological efficacy [1]. The former avoids false positive results, whereas the latter, false negative results. The former may thus be designated as a nontargeted screening without phenotypic information, and the latter, an end-point-specific screening. With the completion of whole-genome sequencing projects, a large paradigm shift in toxicology is that the nontargeted screening enables the prediction of a potential of "no hazard". Thus, it is clear that the whole genome sequencing project potentially should open a new era of toxicology for it to be reconceptualized as a far more *real* and predictable science than previously considered.

The microarray and gene chip technologies applied in toxicology are called "toxicogenomics" (="toxicological transcriptomics") [1]. Toxicogenomics may contribute to the elucidation of the toxicologic mechanism (inductive toxicogenomics), and to the prediction of various nontargeted toxic phenotypes only on the basis of the similarity in gene expression profiles without requiring annotation for neither genes nor chemical characteristics (deductive toxicogenomics). The former inductive toxicogenomics is supposed to define biological markers represented by an unknown gene profile; consequently, markers based on proteomics. On the other hand, the latter deductive toxicogenomics is supposed to predict various possible toxicologic phenotypes even without informative annotation. The former is supposed to contribute to specifically defining targeted toxicity and the latter untargeted toxicity by the combination of expression profiles which leads eventually to the identification of a borderline of "nothing". The predictability of the latter strategy can be enhanced using a database that is a combination of both the above-mentioned inductive and deductive databases. These are analogous to the clinical use of genomics for human tissue

samples and clinical data informatics applied to the diagnosis of diseases, analysis of responses to treatment, and consequent prognosis in each patient [2-4]. Such medical and medicinal information from genomics (cf. SNPs) can be a "custom-made" personalized protocol. Furthermore, the newly established methodology also enables SNP-oriented human ecotoxicological risk evaluation. Toxicogenomics can be categorized into endpoint-specific screening for identifying pharmacologic-specific toxicity, mechanism-based targeted screening, and toxicological profiling, which is a nontargeted screening without phenotypic information, which is the ultimate predictive toxicogenomics, the narrowest meaning of reverse toxicogenomics. The last one requires the accumulation of a large database, whereas ready-made expression arrays for the first and second categories are commercially available which focus on the first and the second applications, for example, chips for metabolic enzymes, such as CYP450s, acyltransferases, and sulfotransferases; growth factors and receptors, including IGFs, interleukins, NGTs, TGFs, VEGT, and nuclear receptors, such as retinoic acid receptor, retinoid X receptors, and PPARs.

Toxicogenomics does not supplement or serve as an additional source of information for conventional toxicology and toxicologic pathology. It is a methodology that elucidates toxicological concepts that are new or different from the established conventional toxicological concept. A dose-response relationship may be one typical example to consider. In conventional toxicology, a dose-response relationship continues from the NOEL (or NOAEL) dose to the maximum or plateau dose at which an animal shows the endpoint phenotype, including death. In toxicogenomics / transcriptomics, different doses may also show a continuous increase in gene expression level similarly to that obtained by conventional toxicologic testings. However, in many cases, different combinations of expression profiles are observed with an increase in the dose. Indeed, when one examines the expression of genes with an increase in 17-beta-estradiol dose in the Venn diagram, the percentages of common genes expressed from a low dose of 0.001 microgram to a high dose of 1.0 microgram, are 9.4% of the total number of expressed genes and only 1.2% of the total number of genes examined [5]. Thus, it is clear that gene expression level does not always seem to increase or decrease with an increase in the dose; but rather, different doses provide different gene expression Vprofiles. Consequently, the dose-dependent profiles of various expressed gene combinations at each dose per se can be a distinct "bio-marker" of the dose. Extrapolatability from '*in vitro*' to '*in vivo*' data, and the possibilities and limits of extrapolated data should also be considered. The activities of cellular membranes such as those of ion channels and ion pumps, the inhibitory effect of uncoupling oxidative phosphorylation, and the inhibition of ATP turnover by redox cycling are examined using commercially available *in vitro* chips. However, the possible toxicities associated with developmental and morphogenetic anomalies do not seem to be sufficiently predictable when using an in vitro cell culture system. On the contrary, the use of a fraction of stem cells, such as embryonic stem cells, hemopoietic stem cells, and neuronal stem cells, is supposed to provide useful information even in the case of using an *in vitro* system. To elucidate the molecular signatures (expression profiles) of hematopoietic stem/progenitor cells, it is important to characterize the expression profile of each stem cell subcompartment of a bone marrow cell that is separated into its appropriate fraction as well as to characterize the differentiation from stem cells to progenitor cells to the terminal differentiated cell fraction. Thus, before microarray analyses, obtaining the expression profile of the specific fraction of hematopoietic stem cells provides essential information useful in future microarray analysis. Homologous disease entities across animal species are rather well known. In the murine model of Hutchinson-Gilford progeria, a point mutation of lamin A shows exactly identical behavioral and cellular phenotypes [6]. Various c-kit mutations in humans and comparable mutations in mice and rats are assumed to reveal similar phenotypes such as anemia and infertility. These lines of evidence imply a future possibility of extrapolation in combination with molecular taxonomic gene profiling.

Key molecules participating in similar physiological responses and toxicological responses are presumably comparable, and their functions seem to be bidirectional. The same oxygen reactive species, on one hand, contribute to xenobiotic activation, but, on the other hand, they

induce cell and tissue damage due to oxidative stress. Apoptosis-related genes are essential for morphogenesis during fetal development, but the induction or suppression of apoptosis after cells and tissues injured by xenobiotic exposure is an essential biomarker of adverse effects. These dual functions of key molecules as well as gene expressions imply that the physiological and toxicological responses may be a "continuum", which has never been observed by a previous single testing method. The genomics / transcriptomics data may elucidate a new molecular border between physiological and toxicological responses. The use of a gene knock-out technique or gene overexpression animals is particularly interesting, since expressed phenotypes may shift along the scale of the continuum for either exaggeration or attenuation, which makes the interpretation and mechanism of xenobiotic responses clearer.

We may have to pay specific attention to the following at this moment. How can one define a specific mechanistic interpretation of the expression profiles for each discontinuous independent parameter. How can one define a possible predictability of gene expression profiles not only for the group whose data were compiled for the establishment of database, but also for the group whose data were not incorporated into the database, that is, unknown compounds. These unknown compounds should be the focus of future toxicogenomics studies.

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VOLUME I

環境ホルモンの最新動向と測定・試験・機器開発

Recent Aspect of Endocrine Disrupters

－Measurement,Examination and Equipments－

監修：井口泰泉 *Supervisor : Taisen Iguchi*

シーエムシー出版

序章　化学物質と健康－低用量問題

井上　達*

1　生体と外界との相互作用

1.1　生体反応の限度幅

生体に対する化学物質の作用は，その生物がその対象物質と“遭遇”するにあたって，どれだけそれに応じた生理機能を備えているかにかかっている。自らの腸内細菌の産生するテトロドトキシンから毒性影響を受けることのない“ふぐ”自身のナトリウムチャンネルの特異な適応はこのことをよく示している。おそらく生物は悠久の昔から蓄積した体験をもとにして，外界・周囲に適応した機能を発揮しているのであるが，他方そこに備わった機能を越えた負荷に堪えることはできない。

こうした対応力の限度幅に対して許容量と呼ぶことがあるが，この呼び方はいつも正しいとは限らない。本節では一見そうした限度の範囲内に見える“possible-risk”を取り上げようとしているが，トキシコロジーはいまこうした“possible-risk”を生体が許容しているか否かの判断の難しさに直面している。ここではこの限度幅をさしあたり恕限度と呼ぶことにしよう。一般論としては生物には確かに極限の負荷に対して適応する“可塑性”も備わっており，先にみた機能的適応もその賜物に他ならない。しかしそれは長い時間軸を以って認識される次元の大きく隔たった問題であり，現時点での化学物質と生体の調和のとれた健康的な相互関係を探求する次元の問題とはいえない。

1.2　“適応反応”と傷害性

けだしトキシコロジーでは，生体影響のどこまでが適応的生体反応で，どこからが障害性変化（ここでは傷害性も同義）であるかの分界点を見定めることが課題となる。そしてその中で恕限度の占める位置も課題である。しかし截然としたその切り分けはしばしば困難なほか，それらは相互に重なり合っている面もあるので，驚くほどに適切な方法論のないことに気づく。つまりこれは新しい課題なのである。

例えば生体反応の限度内，ホメオステーシスの範囲内の変化であれば，それは生理的な変動で

＊　Tohru Inoue　国立医薬品食品衛生研究所　安全性生物試験研究センター　センター長

あり生体への障害性はないものとする見方はしばしば見受けられる。しかしながら本書で取り上げられようとしている内分泌撹乱現象などでは特に，そしておそらくもっと一般的にも，そうしたホメオステーシスの捉え方には多分に疑念が生まれつつある。この点には生物学とトキシコロジーの認識のズレもあるように見え，内分泌撹乱問題の本質もここに焦点がある。ある試験法である現象が見え[1]，他の異なった試験法でそれが見えなかった[2]，といった議論があった。果ては「科学的に」どちらが正しい，正しくないといった議論もなされた。この問題は新しい課題に該当しているので，これを混乱ないしは矛盾ととらえる人々も見られたが，本質は，多分に双方とも正しかったということに収束してゆくのではないかと考えている。

1.3 薬理と“毒理”のcontinuum[3]

化学物質と生体の相互作用，健康の保持を考えると，生体は，外界物質（の濃度）との調和のとれたバランス上に健康を維持していることが伺われる。様々の自然界の物質はもとより，紫外線や可視光のような物理的要素からはじまって，量的調節そのものは“必ずしも”自由にならないながら時間などの要素も同様の生体作用因子としてとらえられる。生体と物質の相互作用を，横軸を反応の時間軸に取った場合の種々の例を薬理学的指標と毒理学的指標を相対的に示すと図1のようになる。そこでは外界物質は，過小に過ぎれば生体の発達維持に支障を来し，過大に過ぎれば逆の面から生体障害（傷害）を引き起こす。いま生体に対する負荷からの回復という視点で考えると，“休養”のもたらす生体作用はある一面での時間軸に対する負の方向への制御ととらえることもできる。ここで人類が作り出す無機・有機の化学物質に対して生体がどのような位置関係を形成しているかについての認識も，同様の視点から理解されるわけであるが，これら

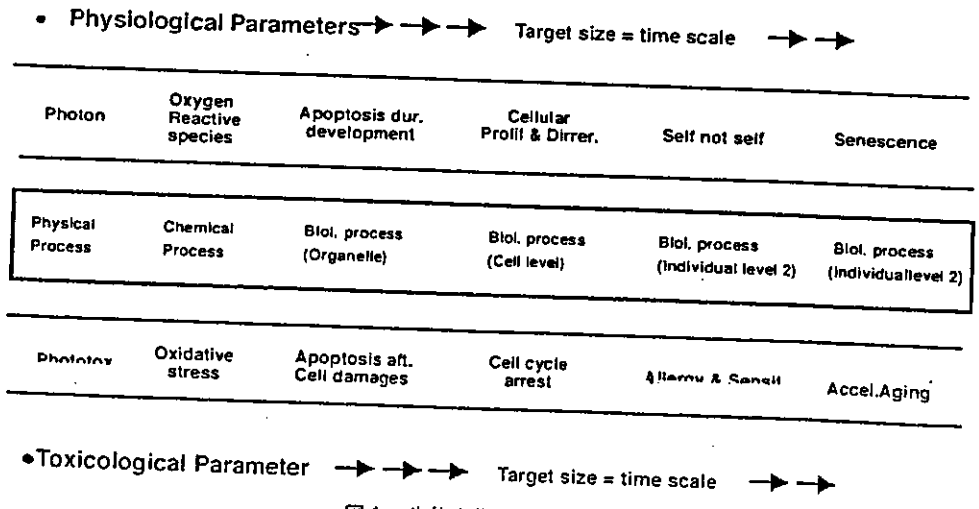

図1　生体と物質の相互作用

については同時に，生物の進化の長い歴史から見ればあまりにも経験の浅い領域に属しており，“未知”の事柄も少なくない。

1.4　恒常性の範囲内のリスク

生体には，獲得された平衡状態の維持機構が備わっていると考えられ，これはホメオステーシス（恒常性）と呼ばれるが，その背景では多次元的でネットワーク状の相互作用・拮抗関係にある様々のモメントのたえざる平衡調節が働いている。こうした関係の中では微量の物質作用は緩衝効果によってうち消されるので，これへの反応は通常の観察方法では検出されないことも知られている。観察されない認識下での事柄の生体への影響の有無や，通常観察されない事柄が生体の特殊な状態下で影響を及ぼす可能性の如何ということになると，これまで無視し得るものと判断されてきたので，当然未知の事柄が少なくない。そこでにわかに注目されているのがここに取り上げる「低用量問題[4]」である。農薬，工業用化学物質などの中に折に触れて見いだされる，ホルモン様の生体作用をもついわゆる内分泌撹乱性化学物質（環境ホルモンは俗称）は，まさにこの低用量問題を焦点としている。そこでは，野生生物の雌化現象や群集単位の縮小，ヒトでの生殖腺の異常あるいは腫瘍発生の増加などが危惧の対象として取り上げられた。結果として，それら環境中のホルモン様生体作用物質（例えば農薬，工業用化学物質）と生体（例えばホルモン受容体）との低用量レベルでの相互関係が問題の本質となっているものとの理解に至っている。これらの諸点についての参考には，米国National Research Councilの“Hormonally Active Agents in the Environment.”(1999)[5]や，WHO/IPCSがまとめたGlobal Assessment of the State-of-the-Science of Endocrine Disruptors.(2002)[6]などがあるほか，小著[7]も参照されたい。(http://www.ehp.niehs.nih.gov/who/)

2　低用量作用への認識

2.1　はじめに

毒性試験とは，障害性（ここでは傷害性も同様）限度試験であり，障害性の観察される限度を見極めることによって，その限度以下の用量における安全性を担保しようとするものである。もしこの前提が崩れるならば毒性試験による安全性の担保は，別の方法によらざるを得ないが，低用量問題は，そうした一環として登場した。このものは，①閾値の有無　②相乗性・相加性の有無，そして，③高用量からの外挿性の可否，反応の線形－非線形用量相関問題，などの諸点に分けて問題提起された。しかし実際にはこれらは相互に連関したひとつの問題である。反応性が線形用量相関を示すことが確かであれば，高用量から直線外挿性に低用量反応が想定可能であり，

低用量域に閾値があれば実質的には相乗・相加問題は発生しないからである。これらについてトキシコロジー領域に個々の具体的なデータは必ずしもなかったかも知れないが，種々の生物学的事象からくる生物学的蓋然性からみると，これらの命題の否定はもとより単純な事柄ではなかった。2000年10月，米国EPAは，ノースカロライナ州で，従来求められてきた無作用量（NOEL）や無毒性量（NOAEL）よりも低い用量域[4]で，いま内分泌撹乱問題で対象となっているようなパラメータに該当する新たな影響が観察され得るものかを問う「低用量問題に関するワークショップ」を開催した。その記録は，EPAのwebsite[4]に紹介されているのでここではふれないが，この会議以後，少しずつ低用量作用に関連する報文がでてきた。それらの諸説に収斂の気配は見えないが，双方にある方向性が認められるので，いずれそれらを整理する機会も近いものと考えられる[7~21]。

2.2 閾値の有無

閾値の有無に関する証明は実質的には生物統計学的に用量相関のモデル型から導き出すことになる。現象面からのそれには，例えば子宮肥大試験でのリガンドの用量に応じた子宮の肥大変化がロジスティックないしはシグモイド・カーブを取ることを以て知られる。因みにロジスティック・カーブの無閾値性はそれ自体では決定論とはならないが，低用量域で限りなくX軸に漸近するという意味で無閾値性を示唆している。EPAのEarl Grayは，抗アンドロジェン作用を持つ物質の種々の雌化指標が同様のロジスティック・カーブもしくはS字状曲線をとっていて，調べた限りでベースラインレベルまで接近したと述べている[22,23]。内分泌撹乱化学物質の疑義のある物質の多くがリン脂質からなる細胞膜をたやすく透過すること，従って，受容体1分子と化学物質1分子が反応するものと考えると，反応性は充分に低用量域に達することにならざるを得ないことなどがこの無閾値仮説の原点であった。事実，ホメオステーシスの環境を切り離した実験系では，*in vivo*試験でさえも極めて低い用量で様々の反応が生ずることがすでに知られている[24]。十分に低用量の領域でのリガンドの受容体との会合は当然確率的に低くはなるので，近年発がん性領域でも用いられる“practicalな”閾値はあるものと考えられる。

2.3 相乗性・相加性の有無

この問題に該当するデータとしては，かつてSoto[25]が複合アッセイ系確立の可能性を論じた報告が原点になると思われるが，この課題に真正面から取り組んだという意味で，最近注目されるのは，ロンドン大学のKortenkampのグループによる相加性に関する報告である[26~28]。彼らの一連の報告は，報告者らの文中にあるような相乗性（synergy）を意味しないが，明らかな相加性（additives）を確認したという意味でその結論は重い。先のE.Grayもvinclozolinと

procymidoneの相互作用が相加的であったとしている[29]。

2.4　反応の線形－非線形用量相関問題

この問題に関するデータは，従来のNOELやNOAELなどよりも低用量で何らかの変動パラメータが観察された，という形で間接的に示される。先のE.Greyは，vinclozolinで，このものの抗アンドロジェン作用が従来の無作用量レベル（数千mg/kg）より低いレベル（100～200mg/kg）で肛門・生殖突起間距離の短縮など様々のパラメータに雌化傾向を生ずることを報告した[22,23]。Bisphenol Aに関連したデータもこのところ数多く認められる。九州大学の粟生（Aou）らによれば，Wistar系の妊娠ラットへのBisphenol A 1.5mg/kg（NOAELは50mg/kg）を投与し，仔の成育後のオープン・フィールドテストにおける行動と，脳の青斑核（locus ceruleus）の小型化など，雌化傾向が見られたと報告している[30]。なお，こうした行動観察については，Grayらも，anti-androgenic chemicalでの結果を追加している[31]。わが国の環境省では，この低用量影響を検出する試験法の開発研究の一環として，改良一世代試験の検討を進めている[32]。その中で，di-cyclohexyl phthalateによるF1世代における8，40μg/kg/dayでのERαmRNAやARのmRNA発現の亢進（従来のNOEL/NOAELは500mg/kg/day［肝重量増加］）やdi-2-ethylhexyl phthalateによるF1世代における50μg/kg/dayでの血清FSHの上昇（従来のNOEL/NOAELは100mg/kg/dayでの肝重量増加）などを観察し，従来のNOEL/NOAEL以下のレベルで，種々のパラメータの変動の見られることを明らかにしている。環境省プロジェクトの低用量における変動パラメータの中にホルモン受容体の遺伝子発現が散見されることは，前節での考察と符合して意味がある。

3　今後の方向性

3.1　低用量問題とChildren's program

低用量問題を通覧すると，これが無視できない生物学的蓋然性を持つことが分かるが，具体的なデータの多くは胎生期間中の形態形成期や，新生児の急激な発育期に関連したものであることに気づく。このことから見ても，内分泌撹乱物質問題そのものが胎児・新生児を含む小児の問題（Children's program[33]）の重要な柱となってゆくことは疑いない。ヒトでの現存疫学データが充分な役割を果たしていない現状にあっても，今日までの結果が示す内分泌撹乱問題の本質に関わるchildren's programの生物学的蓋然性は，明らかに高いと考えられるからである。