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Toxicogenomics

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Introduction:

Toxicogenomics - a new paradigm of toxicology

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Summary. Molecular biology has enabled the elucidation of biological subjects with bilateral strategies, namely, an inductive approach and a deductive approach. Along with the development of the mouse whole-genome sequencing project, it has enabled elucidation of the science bilateral interrelationships between the toxicological phenotypes related to particular toxicants and expression profiles of pertinent genes induced by exposure to toxicants. While a conventional inductive approach permits exploration of the toxicological mechanism by cloning genes and analyzing gene and protein expression during the course of chemical exposure, the newly developed deductive approach potentially permits the elucidation of the toxicological phenotype(s) through gene expression.
Microarray technology has dramatically changed the time course of drug discovery in new drug development. Potential therapeutics can be screened for thousands of endpoints indicative of efficacy and adverse toxicity at one time using the microarray technology. Simultaneously, the same technology can be used to explore unique genomic "expression fingerprints", which can be used to group the biological effects of chemical actions at a various doses, time intervals, or target tissues, in a variety of animal species, into profiles as the bases of gene expression. Accumulation of the expression profiles (here and elsewhere) of whole genomes for reference chemicals for a variety of treatment conditions permits the establishment of an informatics profile (here and elsewhere) for reverse toxicology, which is conversely supposed to predict the toxicological phenotypes solely by analyzing gene expression. This translational introductory oversees the future prospects of how microarrays can be used in applied toxicology.

Key words. Toxicogenomics, DNA microarray, reverse science, reverse genetics, reverse toxicogenomics

DNA microarrays

As an introductory keynote to "Toxicogenomics", a discussion on what toxicogenomics can offer to conventional toxicology is given here in this

paragraph. Toxicogenomics is based on DNA microarray and DNA chip technologies that are similar to those in other genome science fields (Lovett, 2000; Hamadeh et al., 2001; Storck et al., 2002) i.e., the DNA microarray fixed with cDNA by a DNA spotter, and hybridized with fluorescence-labeled cDNAs from tissue samples (Schena et al., 1995, 1996), and the DNA chip, on which a number of oligonucleotide probes are photolithographically synthesized, followed by hybridization of biotiny lated cDNAs from samples (Fodor et al., 1993). Originally, DNA microarray and DNA chip technologies have been used to analyze a large number of gene expressions, and thus, have been applied to such functional genomics fields as transcriptomics (Storck, et al., 2002) pharmacogenomics (Lloyed A, 2000), mutagenomics (Aardema and MacGregor, 2002), oncogenomics (Herrmann, et al., 2001), pathogenomics (Liefers, et al., 2001), and predictive diagnostic medicine based on clinical prognosis (Nakamura, 2001), and specifically, the latter DNA chip technology is a potentially powerful tool for identifying DNA sequences, thus, such inductive information has been applied widely in the research for single nucleotide polymorphism, SNP, in a variety of drug-metabolizing enzymes, etc., to establish an individualized "tailor-made pharmacology".

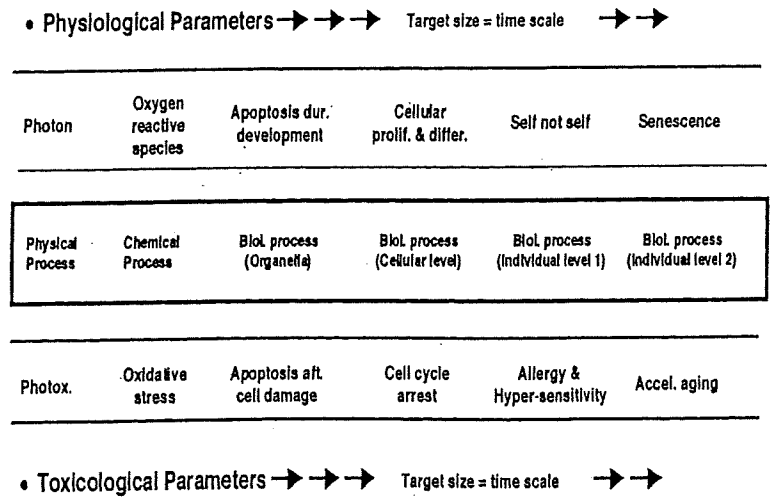


Fig. 1. Physiological parameters shown in the upper row vs. toxicological ones shown in the lower row along with the increase of participating target masses or time scales.

Microarray and /or DNA chip technologies applied in toxicology are called "toxicogenomics". Toxicogenomics can be applied bi-laterally, either inductively or deductively. Deductive approach of toxicogenomics shows a great, unexpected paradigm shift from conventional toxicology.

Toxicology and toxicogenomics

Before describing an over- view of toxicogenomics, what toxicology is, namely, its definition, entity, and scientific bases, should be reviewed first. Toxicology is an interdisciplinary area between biology/medicine and chemistry/physics. Key molecules participating in physiological responses and toxicological responses are presumably comparable (**Figure 1**), implying that physiological responses and toxicological responses may be a continuum. Pharmacology involves the identification of something available; on the other hand, toxicology involves the identification of not only the mechanism of toxicity but also clarifying a border of "nothing", i.e., NOEL, "no observed effect level", and/or NOAEL, "no observed adverse effect level". The goals of toxicology are to predict the effect of potential hazards on human health effects, and to identify the mechanism of toxicity, NOEL and/or NOAEL. In this regard, toxicogenomics is supposed to clarify comprehensively the border of "nothing". Although a prototype of "toxicogenomics" was developed in 1997 (Heller et al., 1997) to identify specific toxicological phenotypes, such as oxidative stress inducers, drug- metabolizing chemicals, and cell-cycle-specific modulators, comprehensive toxicogenomics became possible after the whole-genome sequencing project was accomplished in 2001. Because of the completion of the whole genome sequence, finally, the toxicology to predict "nothing" became possible.

Birth of reverse science & toxicology

In 1988, a new era of mouse genetics, reverse genetics, was started by generating the first knockout gene for mammalian species, murine int-2, by the group of Mario Capecchi's (Capecchi et al., 1988) and then Elizabeth Robertson's (Schwartzberg, et al, 1989). Thereafter, molecular biology has enabled the elucidation of biological subjects by bi-directional strategies, forward and reverse ones, i.e., the inductive and the deductive approach, respectively, where not only genes that possess a particular expression phenotype have been cloned by forward genetics, but also a number of genes of which functions were not known have been uncovered their function by reverse genetics, i.e., knockout technologies. The history of genetics teaches such bilaterally alternating strategies to strengthen scientific power. Thus, it is speculated that the inductive toxicology and deductive toxicology may complement each other.

Along with the development of the mouse whole-genome sequencing project, such bi-directional strategies for analysis became possible also in toxicology; the toxicologic phenotypes of particular toxicants and the expression profiles of pertinent genes reacting with the toxicants. While the inductive approach permits exploration of the toxicological mechanism by analyzing gene and protein expression during the course of toxicological testing, the deductive approach

permits prediction of the toxicological phenotype(s) solely by analyzing the gene expression. Microarray and/or DNA chip technologies have enabled the survey of a large number of gene expressions after exposure to a toxicant. Both inductive and deductive approaches have enabled application of DNA chip and/or the microarray in toxicological analysis, i.e., "toxicogenomics". Toxicogenomics enables exploration of the toxicological mechanism by analyzing a large number of gene chips inductively, and opens a new era of reverse toxicology, which is supposed to predict possible toxicologic phenotypes by distinguishing the expression patterns of particular genes from accumulated expression profiles. The DNA chip and the microarray technologies for the identification of specific toxicity groups are commercially available already, e.g., metabolic enzyme inducers, growth factor & receptor-mediated transducers, xenobiotic ligands for nuclear receptors, stress-response-gene modifiers, and cell-cycle regulator modifiers.

Reverse toxicology

Similar to reverse genetics, reverse toxicology is supposed to identify toxicological phenotypes solely by examining their expression profiles. Such deductive use of microarray technology for toxicology is called "Reverse Toxicogenomics", where it is expected to predict toxicological phenotypes solely by analyzing whole gene expression (**Figure 2**). This technique is requires a

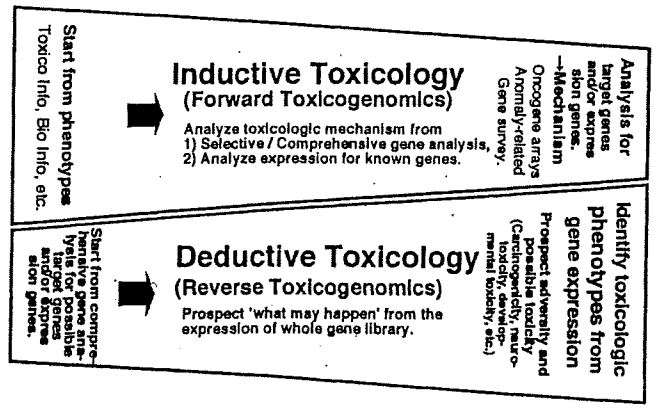


Fig 2. Structures of inductive toxicology vs. deductive toxicology. Former starts its analyses from toxicologic phenotypes toward the mechanism, whereas, the latter focuses in identigying toxicologic phenotypes solely from the gene expression profiling.

minimum number of animals, or even samples of in vitro-cultured cells after a relatively short period of exposure to a potential hazardous testing materials. The predictability by reverse toxicology depends upon the number of gene expression profiles accumulated, the number of phenotypes differentially linked to the gene expression profiles, and informatics linking such gene expressions and the phenotypes.

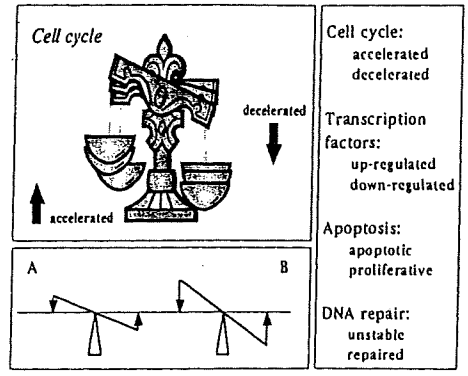
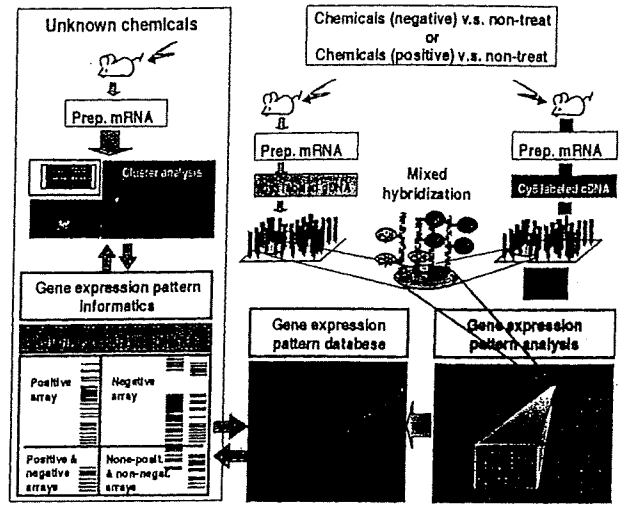


Fig. 3. Counter balancing gene expressions behind the homeostasis Visualization of different oscillatory balances A and B (left bottom).

At this moment, reverse toxicogenomics is still a theory. However, a variety of testing methods in toxicology will be replaced by reverse toxicogenomics, eventually. This strategy has the following advantages: it reduces the number of test animals, and the test period, and adopts simpler techniques by using established expression profile as new biomarkers rather than sophisticated methodologies requiring skill and experience. Furthermore, a specific proteome chip, expressing a series of specific genes, is supposed to function as "reverse proteomics" through a series of processes such as sample preparation, 2D gel electrophoresis, and mass-spectrometry for image analysis (Zhu et al., 2001). To set up an endpoint where NOEL or NOAEL exists, a traditional toxicology has been applied to incorporate something " invisible borders". Invisible borders are, in conventional toxicology, based on at least two major limitations: one in an endogenous factor(s) and the other in an exogenous factor. The former, for example, is hidden behind homeostasis, and the latter, for example, is behinbd a technological limitation. As shown in **Figure 3**, most living animals exhibit homeostasis between two (or more) counter- balancing vectors such as oxidation & reduction, apoptosis & anti-apoptosis, and acceleration & deceleration of cell cycle regulation. Since the counter-balancing counter-directional homeostasis, it appears static and one may not recognize the differences between one homeostatic stage, balanced at a low energy stage, to the other stage, balanced at a high energy stage (A and B in Figure 3). It is far more important to note that stage B is generally more risky. Toxicogenomics is expected to disclose such hidden homeostatic balances which are undetectable by conventional testing systems. The latter, an exogenous factor in a technological limitation, may be based on such resolution limit of light-microscopes, spectrophotometers, etc., and all



technologies exhibit their resolution point, i.e., "invisible barrier". Toxicogenomics also has a limit in terms of technical sensitivity; however, it may overcome presently available resolution limits in many ways, and hopefully identify a possible specific toxicological profiling.

Fig. 4. Practical toxicity-predicting system based on the gene expression microarray.

Practical approach

A sample of a chemical toxicity predicting system is shown in **Figure 4.** Mice are treated with, or without, a known toxic reference chemical (TRC), or treated with, or without, a known non-toxic reference chemical (NTRC). Then, the

Table 1: Possible toxicologic endpoints tested by in vitro or in vivo resources

Possibility of in vitro test	Toxicity Endpoint(s)
No [yes]*	*Morphogenesis, developmental anomaly*
No	*Identifying tissue-specific toxicity*
No/yes	*Epigenetic carcinogenesis (as modification of gene expression)*
	Metabolic activation
No	Hepatic activation leading to multi-tissue damage
Yes	Tissue-specific activation
	Receptor-mediated events
Yes/no	Neuronal tissue
Yes/no	Steroid hormonal tissue
Yes/no	Ah-receptors
Yes	*Cytotoxicity*
	Membrane activities
Yes	ion channels, ion pumps
	Inhibition of biochemical process
Yes	Uncoupling oxidative phosphorylation, inhibition of ATP production by redox cycling initiation to produce ROS
Yes	Oxidative phosphorylation to inhibit ATP production
Yes/no	Alteration of calcium homeostasis

*[yes]: Limited possibility at this moment; e.g. whole embryo culture.

messenger RNAs are extracted, and visualized with red color marker, cy3, for overexpression or with green color marker, cy5, for down-modulation. These color-labeled mRNAs will be processed into a competitive mixed-hybridization in a high-density hybridization array. Expression patterns are informatized in many ways. Along with accumulation of data to establish informatic profiles, specific gene clusters for TRC, NTRC, and those positive for both TRC + NTRC, andnegative for both TRC + NTRC, can be established. These databases can be compared with an expression profile that will be obtained from unknown chemicals (left box in Figure 4).

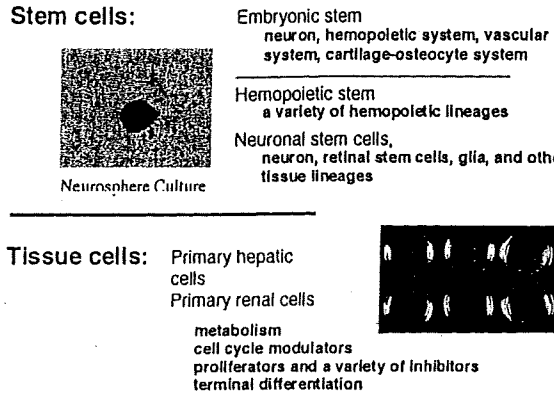


Fig. 5. A variety of in vitro resources for toxicologic gene expression array. Different cellular function for microarray analyses between stem cells and tissue cells. See text.

The technique requires only a limited number of animals, or even with cultured cells, in vitro, after relatively a short period of exposure. Depending upon the endpoints of toxicity aimed to focus on, even materials from in vitro culture may work efficiently (**Table 1**). As shown in Table 1, activities of membrane such as ion channels and ion pumps, the inhibitory effect of uncoupling oxidative phosphorylation, and the inhibition of ATP turnover by redox cycling, may be identified by the microarray. Possible toxicity related to developmental anomaly and morphogenesis (top of Table 1) may not be predictable by the use of in vitro cell culture; however, as seen in **Figure 5**, an in vitro system, for example, an embryonic stem (ES) cell, may predict some possible adverse effects of toxicity on the morphogenesis. Consequently, ES cells as well as hemopoietic and neuronal stem cells are particularly powerful tools for identifying the effect of toxicity on not only proliferation but also differentiation. Actually, one ES cell potentially corresponds to one individual; therefore, observing a microarray of ES cells may correspond to observing several millions of mice at the early developmental stage. Hepatic, renal and other types of primary cultured cells are limited but useful for observing such a variety of metabolic modulators, cell cycle regulators, and cell proliferation inhibitors and/or stimulators, in primary hepatic cells.

New paradigm of toxicology

Toxicogenomics, specifically reverse toxicogenomics, is about to open a new paradigm of toxicology from, at least, five aspects: first, the merging of such scientific borders as physiology and toxicology (also pharmacology and toxicology); second, a paradigm shift from "analog science" to "digital science"; third, visualization of hitherto unknown oscillatory changes behind homeostatic balances; fourth, comprehensive inter-species extrapolation; and lastly, a paradigm shift from inductive toxicology to deductive toxicology. As discussed previously in the first paragraph of this chapter, when we compare the aims of physiology, and toxicology, we find that they face opposite directions; however, participating molecules, seen in Figure 1, are presumably shared each other, physiologically as well as toxicologically, thereby implying that physiologic responses and toxicologic responses may be a continuum. In contrast, gene expression may not be continuum along with the dose response relationship, although a simple linear dose-response curve is generally accepted in the traditional toxicology. It appears to be clear that a different dose gives a different gene expression profile, in other words, the expression is expected to show not an analog change but a digital one, and a different dose behaves as a different chemical in the microarray. Although simple linear dose-response curves seem to apply in many cases, toxicological parameters may change discontinuously based on the genomic expression. This may be an advantage on one hand, because an appropriate array profiling of toxicologic responses can be eventually identified. On the other hand, there may still be a long way to go before reaching the final goal of defining the specific toxicologic array profiling for appropriate toxicologic phenotypes. The concept of safety borders, such as NOEL and NOAEL, may likely be re-established likely by means of such specific safety profiles. Visualization of invisible homeostatic balances is shown in the Figure 3. As was mentioned in the paragraph on oscillation strength, an additional new concept of "risk" may be re-established. Comprehensive 'interspecies'-extrapolation may be improved by informatics over different species such as mouse, rat, frog (Xenopus), and yeast. Interspecies extrapolation will be dealt with on a theoretical basis using a relatively small number of genes that are known to confer important allelic variations. This would result in better interspecies extrapolation, higher confidence of animal models, reduction in the number of animals needed for testing, shorter testing period, and most importantly, insights into pathways of toxicity and their mechanisms (US-EPA). Reverse toxicogenomics may be supported by the other four paradigm shifts mentioned above, and be used to predict toxicity and establish new concepts in risk assessment methodologies.

By means of toxicogenomics, we will be able to see a new toxicological world behind homeostasis and/or gene expression balance.

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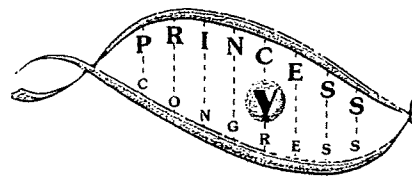

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VOLUME I

POTENTIAL APPLICATIONS OF TOXICOGENOMICS IN RISK ASSESSMENT

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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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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

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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

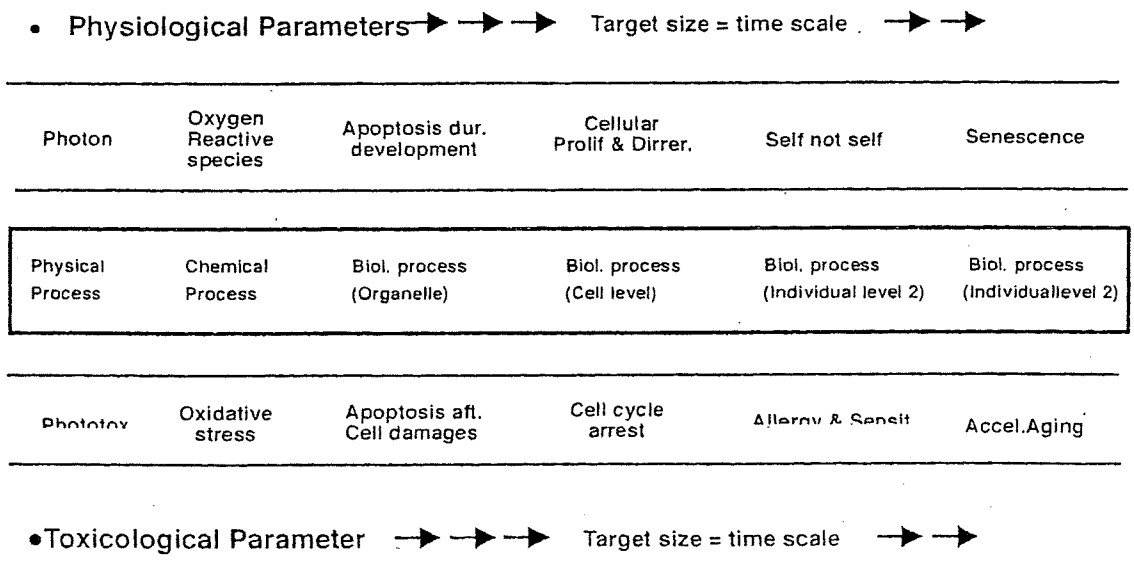

図１　生体と物質の相互作用

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A

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

この問題に関ずるデータは，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。