21. Scientific Implications and Social Impact of Threshold Concept for Genotoxic Carcinogens

Yuzo Hayashi (Japan Health Food & Nutrition Food Association, Japan)

Hayashi discussed the classification of genotoxic and non-genotoxic carcinogens. This classification, however, can not be applied to all instances due to insufficiencies in necessary information. Therefore, the non-threshold concept was introduced exclusively for genotoxic carcinogens and has been adopted in Japan as a basis for regulatory risk assessment. Dose-response studies recently conducted with various genotoxic agents suggest the existence of a threshold. It should be emphasized, however, that a threshold is not a value which can be determined directly from dose-response data. In this context, scientific efforts in support of the adoption of a threshold should be focused on the development of appropriate mathematical models, and the establishment of toxicological concepts. A realistic step towards a paradigm shift from the non-threshold concept is to seek general consensus on the introduction of an appropriate "virtually safe dose" instead of a threshold.

22. Closing Remarks

Shoji Fukushima (Japan Bioassay Research Center)

Fukushima emphasized that evaluation of threshold in carcinogenicity of genotoxic carcinogens is a very important problem in cancer risk assessment and management. Furthermore, various services as well as consumers and industrial workers mutually desire the fast solution of this problem. In the present Symposium, the speakers did the presentations on the matter of risk assessment, risk management and risk communication for free and active discussion as well as exchanging ideas and opinions. Compared to the Symposium organized in two and half years before by Dr. M. Hayashi (NIHS, formerly) and he, in this time more people were gathered and a deeper and mutual comprehension was achieved. It is very important to evaluate the benefit and risk of chemicals on the basis of our latest scientific results and to continue discussion and argumentation on carcinogenic threshold. Furthermore, together with overall look on the problem of threshold, more and more understanding is continuously desired.

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Review

Possible Mechanisms of Practical Thresholds for Genotoxicity[1]

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An axiom in regulatory sciences is that there are no thresholds for genotoxicity of chemicals. It leads to another default assumption that genotoxic carcinogens impose cancer risk on humans without thresholds, i.e., a linear non-threshold model. Therefore, no acceptable daily intake (ADI) is set for food additives, pesticides and veterinary drugs when they have genotoxic and carcinogenic activities. However, humans possess a number of defense mechanisms such as metabolic inactivation, DNA repair, error-free translesion DNA synthesis and so on. These mechanisms may constitute practical thresholds for genotoxicity. Error-free translesion DNA synthesis is a process where DNA polymerases bypass lesions in DNA by insertion of correct bases opposite the lesion and continue replication of whole chromosomes. These mechanisms might have been evolved because organisms from bacteria to humans are exposed to endogenous as well as exogenous genotoxic compounds. In fact, levels of spontaneous mutagenesis are strongly influenced by ability of DNA repair and translesion DNA synthesis of the host cells. Here, I show evidence that DNA repair and translesion DNA synthesis play roles in practical genotoxic thresholds in *Salmonella typhimurium* used for bacterial mutation assays, and discuss future directions of the research on genotoxic thresholds *in vivo*.

Key words: genotoxic thresholds, DNA repair, translesion DNA synthesis, ADI

Introduction

Human chromosome is exposed to a variety of endogenous and exogenous agents (1,2). The most prominent endogenous genotoxic agents are reactive oxygen species (ROS), which are generated as by-products of oxygen metabolism (3,4). These reactive molecules include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen. ROS is also generated in cells by exposure to radiation and chemical carcinogens. Because ROS damages nearby cellular components such as DNA, proteins and lipids in membrane, cells must have evolved multiple defense mechanisms to combat the oxidative stress. Enzymes such as catalase or superoxide dismutase detoxify ROS, and low-molecular-weight scavengers such as glutathione alleviate the toxicity of ROS. Nevertheless, some ROS molecules escape from the defense systems and inevitably damage the biomolecules. DNA repair mechanisms, e.g., 8-hydroxyguanine (8-OH-G) DNA glycosylase encoded by *OGG1* in humans and *mutM* in *Escherichia coli*, remove the damage and convert the modified bases to unmodified ones (5,6).

Another class of endogenous genotoxic agents is alkylating agents such as *S*-adenosylmethionine (SAM) (7). SAM is an S_N2-(bimolecular) alkylating agent and induces 7-methylguanine and 3-methyladenine in DNA non-enzymatically (8). Although 7-methylguanine is formed more abundantly than 3-methyladenine, it is an innocuous modified base. 3-Methyladenine in DNA blocks DNA replication and is cytotoxic. It is estimated that about 600 3-methyladenine residues are formed by SAM in the DNA of a mammalian cell per day (9). Other S_N2-alkylating agents, e.g., naturally occurring methyl halides, induce N^1-methyladenine and N^3-methylcytosine in particular in single-stranded DNA (10). Endogenous S_N1-(monomolecular) alkylating agents such as nitrosamines may induce O^6-methylguanine and O^4-methylthymine, which are mutagenic and toxic lesions in DNA. As in the case of ROS, cells possess a number of defense mechanisms against alkylation damages in DNA (10). O^6-methylguanine DNA methyltransferase (MGMT) directly removes methyl groups from O^6-methylguanine and O^4-methylthymine, and 3-methyladenine DNA glycosylase excises 3-methyladenine from DNA, followed by gap-filling by DNA polymerases (DNA Pols). AlkB in *E. coli* and the counterparts in humans, i.e., ABH2 and ABH3, oxidize methyl groups modified in N^1-adenine and N^3-cytosine, and remove the methyl groups, thereby reverting them into intact adenine and cytosine bases, respectively.

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In addition to endogenous genotoxic agents, human DNA is damaged by exogenous chemical and physical genotoxic insults (11). These include ultraviolet light (UV), radiation, cigarette smoke, polluted air, mutagenic heterocyclic amines, asbestos and so on. The detrimental factors induce bulky DNA adducts, single- or double-strand breaks in DNA. Again, humans possess repair mechanisms against the lesions, such as nucleotide excision repair and homologous or non-homologous recombination, which are responsible for the repair of bulky DNA adducts and strand breaks in DNA, respectively (12). Even when adducts in DNA are not removed, error-free translesion DNA synthesis (TLS) bypasses the damage, thereby reducing the chance of induction of mutations and chromosome aberrations (13) (see below for more detail).

Here, I discuss the possibility that the abovementioned defense mechanisms, i.e., DNA repair and translesion DNA synthesis, may contribute to establish "practical thresholds" for genotoxicity. The term "practical thresholds" is defined as the doses below which no mutations are detectable (14,15). We developed sets of repair-deficient derivatives of *Salmonella typhimurium* TA1535, which is widely used in Ames genotoxicity assay, and used them for demonstration of the practical thresholds for genotoxicity (16–18). I also briefly discuss the *in vivo* (mouse) research on genotoxic thresholds.

MGMT is a Constituent of Oractical Thresholds for Alkylation-induced Genotoxicity

First, we constructed an MGMT-deficient derivative of strain TA1535, namely YG7108, and compared the dose-responses against alkylating agents (16,17) (Fig. 1). MGMT is encoded by two genes in *Salmonella*, namely ada_{ST} and ogt_{ST} (16,19). Both gene products remove mutagenic lesion, i.e., O^6-methy, ethyl, propyl and butyl guanine in DNA, which are induced by a variety of alkylating agents. The alkylating agents used in the study are *N*-methyl-*N'*-nitro-*N*-nitorosoguanidine (MNNG), *N*-ethyl-*N'*-nitro-*N*-nitorosoguanidine (ENNG), *N*-propyl-*N'*-nitro-*N*-nitorosoguanidine (PNNG), *N*-butyl-*N'*-nitro-*N*-nitorosoguanidine (BNNG) and methyl methanesulfonate (MMS). MNNG, ENNG, PNNG and BNNG are different in the length of alkyl chains and MMS induces 3-methyladenine, a cytotoxic lesion, in addition to O^6-methylguanine. The *ada*- and *ogt*-deficient strain YG7108 exhibited superior sensitivity to the genotoxicity of all the alkylating agents used compared to the repair proficient strain TA1535. In particular, the mutagenicity of ENNG and MMS is clear in YG7108 while the mutagenicity is almost completely suppressed in the repair proficient strain TA1535. In the low dose range of MNNG, the mutagenicity was only observed with YG7108 but not TA1535. These results strongly suggest that MGMT is a constituent of practical thresholds for alkylating agents in *Salmonella* strains.

8-OH-G DNA Glycosylase is a Constituent of Practical Thresholds for Oxidation-induced Genotoxicity

Next, we compared the dose responses between $mutM_{ST}$-deficient and proficient derivatives of *S. typhimurium* TA1535 and TA1975, i.e., YG3001 and YG3002, respectively, against oxidative mutagens (Fig.

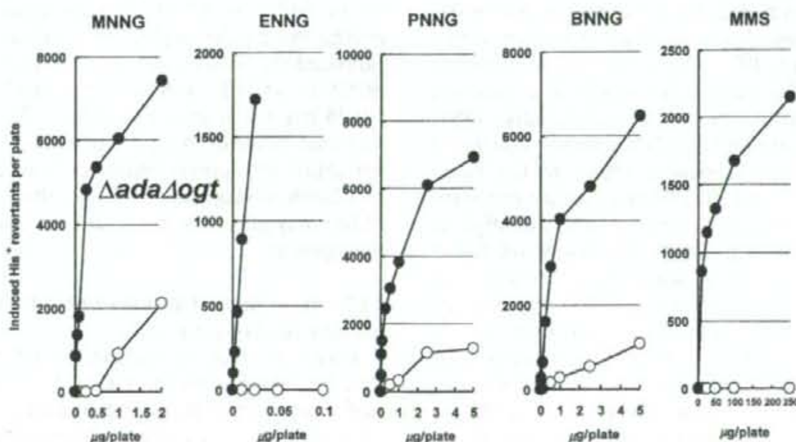

Fig. 1. Dose response curves of *Salmonella typhimurium* YG7008 (*ΔadaΔogt*) and its parent strain TA1535 against MNNG, ENNG, PNNG, BNNG and MMS. Closed circles, YG7008; open circles, TA1535. The assay was conducted as described by Maron and Ames (44), with preexposure of the cells to the alkylating agents for 20 min at 37°C before plating without removal of the alkylating agents. Data are from references (13,14).

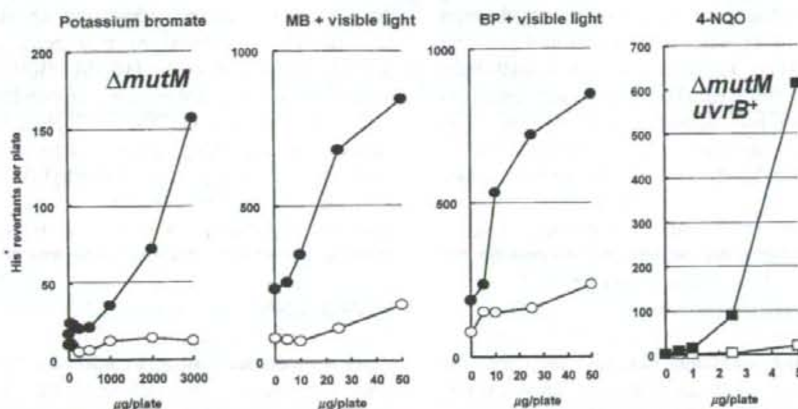

Fig. 2. Dose response curves of *Salmonella typhimurium* YG3001 ($\Delta mutM_{ST}$, $\Delta uvrB$), and its parent strain TA1535 ($\Delta uvrB$) against potassium bromate, MB + visible light and BP + visible light. Closed circles, YG3001; open circles, TA1535. The most right panel shows dose response curves of *Salmonella typhimurium* YG3003 ($\Delta mutM_{ST}$, $uvrB^{+}$) and its parent strain TA1975 ($uvrB^{+}$) against 4-NQO. Closed squares, YG3003; open squares, TA1975. The assay was carried out as written in the legend of Fig. 1. When the mutagenicity of MB and BP was assayed in the presence of visible light, plates were exposed to fluorescent light 15 W lamps at a distance of 30 cm during incubation at 37°C for two to three days. Data are from (18,26).

2). The $mutM_{ST}$ gene encodes 8-OH-G DNA glycosylase in *S. typhimurium* (18). Both YG3001 and YG3002 are deficient in $mutM_{ST}$ but YG3001 is also deficient in functions of nucleotide excision repair ($\Delta uvrB$). The oxidative mutagens used are potassium bromate, methylene blue (MB) plus visible light, benzo[a]pyrene (BP) plus visible light and 4-nitroquinoline *N*-oxide (4-NQO). Potassium bromate is a rat renal carcinogen and induces 8-OH-G in DNA (20). MB is a photosensitizer and induces 8-OH-G in DNA in the presence of visible light (MB plus visible light) (21). BP is a well known genotoxic carcinogen upon metabolic activation, but the mutagenicity was assayed without metabolic activation in this study (22). Instead, BP was activated by exposure to visible light (BP plus visible light). 4-NQO is a genotoxic carcinogen too. Although 4-NQO induces bulky DNA adducts and oxidative lesions (23), the bulky adducts are removed by nucleotide excision repair in the backgrounds of TA1975 and YG3002 and thus the mutagenicity in the backgrounds depends on the oxidative lesion, namely 8-OH-G in DNA. The $mutM_{ST}$-deficient stains exhibited much higher sensitivity compared to the proficient strains (18). The mutagenicity of potassium bromate and 4-NQO was clearly observed in the $mutM_{ST}$-deficient strains, i.e., YG3001 and YG3002, respectively, while the mutagenicity was almost completely suppressed in the proficient strains, i.e., TA1535 and TA1975. Strain YG3001 also exhibited much higher sensitivity against MB plus visible light and BP plus visible light. These results suggest that 8-OH-G DNA glycosylase contributes to establish practical thresholds against oxidative mutagens.

8-OH-G DNA glycosylase is present not only in bacteria but also in humans (24). The glycosylase in humans is encoded by *OGG1*. Interestingly, there is a genetic polymorphism in the human *OGG1* gene (25). We conducted a functional complementation assay where three polymorphic forms of human *OGG1*, i.e., hOGG1-Ser326, hOGG1-Cys326 and hOGG1-Gln46, are expressed in *Salmonella* strain YG3001 deficient in the bacterial $mutM_{ST}$ gene and the mutagenicity of MB plus visible light was assayed with the strains (26). Although human OGG1 proteins suppressed the photomutagenicity of MB, the extent of suppression was different among three polymorphic forms where hOGG1-Gln46 exhibited the weakest suppression (hOGG1-Ser326 and hOGG1-Cys326 have Arg at amino acid 46 and hOGG1-Gln46 has Ser at amino acid 326). The results suggest that each polymorphic form of OGG1 may have different ability to suppress mutations induced by the oxidative DNA damage and also that the genetic polymorphism may affect the practical thresholds for oxidative mutagenesis.

TLS may be a Constituent of Practical Thresholds for Genotoxicity

Recent progress in research on DNA Pols revealed that humans possess more than 14 DNA Pols per cell and about half of them participate in DNA repair and TLS (27). TLS is a process where DNA Pols continues DNA synthesis across lesions (11). If correct bases are inserted opposite the lesions, TLS will reduce the chance of induction of mutations and contribute to DNA damage tolerance. However, if incorrect bases are in-

serted opposite the lesions or skip the lesion, it will induce point mutations such as base substitutions or frameshifts. If no TLS occurs, DNA replication may stall and DNA strands may be broken, which leads to chromosome aberrations. Therefore, TLS is a critical molecular event whether DNA damage is converted to mutations including chromosome aberrations or not. Even in *S. typhimurium*, whose genome size is about 1/1,000 of the size of human genome, there are six DNA Pols (13,28). Five of the Pols are encoded by the genes in the chromosome and the remaining one is encoded by the gene on the cryptic plasmid (29). Moreover, there is an additional plasmid pKM101 in *S. typhimurium* TA98 and TA100, where the *mucAB* genes encoding DNA Pol R1 are present (30). The presence of plasmid pKM101 carrying the *mucAB* genes strongly affects the sensitivity of *S. typhimurium* strains to a variety of chemical. In particular, the mutagenicity of furylfuramide (AF-2) and aflatoxin B_1 can be clearly detected with strain TA100 harboring plasmid pKM101 while no mutagenicity is observed with strain TA1535, the same as TA100 but has no plasmid pKM101 (31). AF-2 is a food additive that has been banned in Japan because of the carcinogenicity in the mice, and aflatoxin B_1 is a fungal toxin that can induce liver tumors in humans. It is supposed that DNA Pol R1 bypasses DNA adducts induced by AF2 and aflatoxin B_1 in an error-prone manner while other six DNA Pols in *S. typhimurium* can not. In contrast, human DNA Pol η is responsible for protection of genomic DNA from mutagenic effects of UV (32,33). This enzyme carries out error-free TLS across pyrimidine dimers in DNA and reduces the chance of mutations induced by UV. Lack of DNA Pol η leads to induction of Xeroderma pigmentosum variant, which is a genetic disease whose patients are highly sensitive to sunlight-induced skin cancer. Interestingly, bacterial DNA PolRI and human DNA Pol η belong to the same family of DNA Pol, i.e., Y-family. Therefore, TLS mediated by Y-family DNA Pols may enhance or reduce the frequencies of mutations, thereby influencing the practical thresholds for genotoxicity.

Both DNA Repair and Error-prone TLS Affect Levels of Spontaneous Mutagenesis

As written in Introduction, chromosome DNA is continuously exposed to not only exogenous genotoxic agents but also to endogenous ones. These endogenous lesions are causes for so-called spontaneous mutations (1). Interestingly, both DNA repair and TLS play important roles in regulations of spontaneous mutagenesis. When ada_{ST} and ogt_{ST} encoding MGMT are deleted in *S. typhimurium* TA1535, the number of spontaneous His^+ revertants per plate increases two- to three-fold (16). Similar extent of an increase in the number of spontaneous revertants per plate was observed in $\Delta mutM_{ST}$ strain, i.e., YG3001 (18). Introduction of plasmid pKM101 enhances the number of spontaneous revertants per plate of strain TA1535 more than five times (31). Both deletions of the repair genes and introduction of plasmid pKM101 exhibit additive effects on the spontaneous mutagenesis. These results suggest that the levels of spontaneous mutagenesis, which may play important roles in determination of threshold levels for genotoxicity, is strongly affected by the ability to repair DNA damage and to bypass lesions by DNA Pols in host cells.

Discussion

In theory, even a single molecule of mutagens could interact with DNA and induce genetic alterations, which might lead to cancer (15). Therefore, it is supposed that there are no thresholds for the risk of genotoxic and carcinogenic compounds and also that even a small amount of such compounds can impose carcinogenic loads on humans. Because of the assumption, no accepted daily intake (ADI) is set for food additives, pesticides and veterinary drugs when they have genotoxic and carcinogenic activities. The assumption is counterintuitive, however, because humans possess a number of defense mechanisms against endogenous and exogenous genotoxic insults. The mechanisms include antioxidants, detoxication metabolisms, DNA repair and error-free TLS (Fig. 3). These mechanisms may suppress genotoxicity and reduce it below the detection limits. In fact, both MGMT and 8-OH-G DNA glycosylase strongly affect the sensitivity of *S. typhimurium* strains for genotoxicity assays, thereby suggesting the possibility that they may be constituents of practical thresholds for genotoxicity. In some cases, however, that linear non-

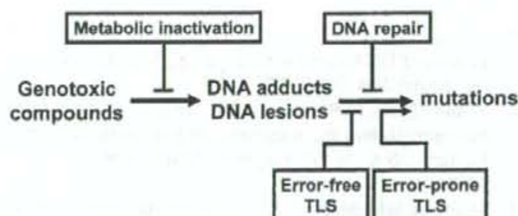

Fig. 3. Possible mechanisms underlying practical thresholds for genotoxicity. Detoxication mechanisms inactive genotoxic compounds. When active genotoxic compounds induce DNA adducts, DNA repair mechanisms remove them, thereby reverting the modified bases into intact ones. Error-free translesion DNA synthesis (TLS) inserts correct bases opposite the lesions and reduces the chance of induction of mutations. Therefore, these mechanisms, i.e., metabolic inactivation, DNA repair and error-free TLS, may be constituents of practical thresholds for genotoxicity. In contrast, error-prone TLS enhances mutations by insertion of incorrect bases opposite the lesions or skipping the lesions. These molecular events lead to base substitutions and frameshift mutations, respectively.

threshold dose response can be observed for genotoxicity even in the presence of wild-type DNA repair (34). Although error-free TLS can reduce the levels of mutations, error-prone TLS has an opposite effects and enhances the sensitivity to genotoxic compounds. Genetic approaches with cells deficient in DNA repair capacity and/or TLS are powerful tools to analyze possible mechanisms underlying practical thresholds for genotoxicity. Since risk assessment of chemical carcinogens is usually conducted with experimental animals, i.e., rats and mice, it is necessary to expand the genetic approaches to *in vivo*. In this respect, *gpt* delta rats and mice may be useful backgrounds to investigate constituents of the practical thresholds (35,36). These transgenic rodents harbor reporter genes for mutations, which enable to identify genotoxicity in target organs of chemical carcinogens (37). So far, *gpt* delta mice have been crossed with a number of knockout mice such as *p53*, *Ogg1*, *Parp-1*, *Atm*, *IL-10* and *Nrf-2* (38–43). It is important to examine which factors, e.g., detoxication, DNA repair or TLS, most strongly affect the levels of practical thresholds for genotoxicity and carcinogenicity *in vivo*. These studies are currently in progress in our laboratory.

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Review

The Concept of "Practical Thresholds" in the Derivation of Occupational Exposure Limits for Carcinogens by the Scientific Committee on Occupational Exposure Limits (SCOEL) of the European Union[1]

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In Europe, there has been a scientific discussion on possible thresholds in chemical carcinogens since the late 1990s. Based on this discussion, the Scientific Committee on Occupational Exposure Limits (SCOEL) of the European Union has discussed a number of chemical carcinogens and has issued recommendations. For some carcinogens, health-based Occupational Exposure Limits (OELs) were recommended, while quantitative assessments of carcinogenic risks were performed for others. For purposes of setting OELs the following groups of carcinogens were adopted: (A) Non-threshold genotoxic carcinogens; for low-dose assessment of risk, the linear non-threshold (LNT) model appears appropriate. For these chemicals, the risk management may be based on the ALARA principle ("as low as reasonably achievable"), technical feasibility, and other socio-political considerations. (B) Genotoxic carcinogens, for which the existence of a threshold cannot be sufficiently supported at present. In these cases, the LNT model may be used as a default assumption, based on the scientific uncertainty, and the ALARA principle may be applied as well. (C) Genotoxic carcinogens with a *practical* threshold is supported by studies on mechanisms and/or toxicokinetics; health-based exposure limits may be based on an established no-observed adverse effect level (NOAEL). (D) Non-genotoxic carcinogens and non DNA-reactive carcinogens; for these compounds a *true* ("perfect") threshold is associated with a clearly founded NOAEL. The mechanisms shown by tumor promoters, spindle poisons, topoisomerase II poisons and hormones are typical examples of this category. Health-based OELs are derived for carcinogens of Groups C and D, while a risk assessment is carried out for carcinogens of Groups A and B. In order to highlight the most important differentiation between Groups B and C, the basic reasoning is given for the six compounds formaldehyde, vinyl acetate, acrylonitrile, acrylamide, trichloroethylene and methylene chloride.

Key words: Occupational Exposure Limits, carcinogens, genotoxicity, mode of action, thresholds, workplace chemicals, SCOEL

Introduction

In 1995, the European Commission has decided (Decision 95/320/EC) to set up a permanent advisory committee with the mandate to propose and justify Occupational Exposure Limits (OELs) and Biological Limit Values (BLVs) for chemical exposures at the workplace (1,2). Since 1998 recommendations for health-based OELs have been issued by the Scientific Committee on Occupational Exposure Limits (SCOEL) (3,4). For genotoxic carcinogens, numerical risk assessments were elaborated, when these were possible on the basis of the available data. For clearly non-genotoxic carcinogens health-based OELs were documented based on established No-Observed Adverse Effect Levels (NOAELs), according to commonly accepted procedures (5,6).

By end of the 1990s, the German "MAK-Commission" proposed a modification of the general procedure, in order to establish health-based OELs ("MAK values") for some additional carcinogens (7). There was no general harmonization of the procedures for carcinogenic health risk assessment in Europe at this time (5). However, there was a growing recognition that carcinogenic risk extrapolation to low doses, which is a pivotal step for setting standards for carcinogenic substances, must consider the mode of action. In Europe, landmarks of the scientific discussion were an *ECETOC-EEMS Symposium on Dose-Response and Threshold-Mediated Mechanisms in Mutagenesis* in Salzburg/Austria (8), results the working group "*Environmental Standards–Dose-Effect Relations in the Low Dose Range and Risk Evaluation*" of the European Academy

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Bad Neuenahr-Ahrweiler (9) and a continuous effort of the EUROTOX Speciality Section Carcinogenesis (10–12). Positions taken by SCOEL on the derivation of OELs for carcinogens considered the scientific discussions (13,14) in Europe and elsewhere and were also presented at various fora. The final strategy has been described by SCOEL in a methodology document and was published in the open literature (15).

Genotoxic *versus* Non-Genotoxic Carcinogens

For risk assessment purposes, there is general agreement to distinguish between chemicals acting through genotoxic and non-genotoxic mechanisms of carcinogenesis.

Non-genotoxic carcinogens (e.g. hormones, tumor promoters, TCDD-like compounds) are characterized by a "conventional" dose-response relationship that allows the derivation of a NOAEL for induction of tumors. Application of an uncertainty factor allows the derivation of permissible exposure levels, at which no relevant human cancer risk is anticipated. The risk assessment approach for non-genotoxic chemicals is similar among different regulatory bodies world-wide (5). Therefore, OELs derived for "true non-genotoxicants" are considered as health-based exposure limits.

For the broad array of *genotoxic carcinogens*, there is the need of further differentiation. Positive effects only at chromosomal level, e.g. aneugenicity or clastogenicity, in the absence of mutagenicity, may characterize a substance that produces carcinogenic effects only at high, toxic doses (16). Such *non-DNA-reactive genotoxicants* include topoisomerase inhibitors (17), or inhibitors of the spindle apparatus or associated motor proteins (18). In such cases, SCOEL agrees to the existence of a threshold (19,20). For some other chemicals, the genotoxic effect may be relevant only under conditions of sustained local tissue damage and associated increased cell proliferation. Formaldehyde (21) and vinyl acetate (22,23) represent such examples, which are explained below. In such cases, the derivation of a "*practical*" threshold (23) seems justified. This denomination is equivalent to the "apparent" threshold as defined by Kirsch-Volders *et al.* (24). Such genotoxic effects may be thresholded, and for substances acting through such mechanisms of carcinogenicity a health-based exposure limit may be set.

For DNA reactive, tumor initiating genotoxic carcinogens (e.g. alkylating chemicals or ionizing radiation) the classical linear non-threshold (LNT) extrapolation appears scientifically sound and, therefore, no threshold can be defined in such cases. Streffer *et al.* (9) have suggested a further differentiation to be made within this group of genotoxicants, also considering chemicals for which there is more uncertainty on their dose-response relationship. In such cases, LNT extrapolations may be used as a default procedure.

Table 1. Development of nomenclature to distinguish types of threshold for carcinogenic or mutagenic compounds*

Author(s)	Ref.	SCOEL Group C	SCOEL Group D
Seiler *et al.* 1977	25	*apparent*	*real*
Kirsch-Volders *et al.* 2000	24	*apparent*	*absolute/real (statistical for spindle poisons)*
Hengstler *et al.* 2003	23	*practical*	*perfect*
Bolt & Degen 2004	11	*practical/apparent*	*true/perfect*
Bolt & Huici-Montagud 2008	15	*practical*	*true/perfect*

*See text for explanation

Types of Thresholds Discussed for Carcinogens

There has been a debate on the nomenclature of different types of thresholds for carcinogenic compounds (see Table 1). The original idea to differentiate between *apparent vs. real* threshold genotoxins dates back to Jörg Seiler (25) in 1977. More recently, Kirsch-Volders *et al.* (24) discussed this issue, proposing definitions for *absolute*, *real* or *biological*, *apparent* and *statistical* thresholds. Hengstler *et al.* (23) distinguished between *perfect* and *practical* thresholds, again based on different types of mechanisms. Basically, non-genotoxic carcinogens were connected with a *real* (24) or *perfect* (23) threshold. A *statistical* threshold (24) has been attributed to mitotic spindle poisons. Definitions of *apparent* (24) or *practical* thresholds (23) are based on the concept that the chemical should cause no genotoxic effect at very low or even immeasurable target concentrations (25). Such *apparent* thresholds have been connected with rapid degradation (toxicokinetics) of the chemical or to other factors that limit target exposures (24).

Taking these concepts together, it has been proposed to basically distinguish between *perfect* and *practical* thresholds. Thus, *perfect* thresholds (23) include both *real* and *statistical* thresholds as defined by Kirsch-Volders *et al.* (24), and *practical* thresholds (23) are equivalent to *apparent* thresholds, as defined by Kirsch-Volders *et al.* (24).

An international scientific discourse on these matters is still ongoing, and the existence of thresholds at very low doses is being discussed even for highly genotoxic compounds like *N*-nitrosamines (26–28).

The Definitions Adopted by SCOEL

Altogether, the aforementioned discussions and developments have led to the adoption by SCOEL of the following four groups of carcinogens:

(A) Non-threshold genotoxic carcinogens; for low-dose assessment of risk, the linear non-threshold (LNT) model appears appropriate. For these chemicals,

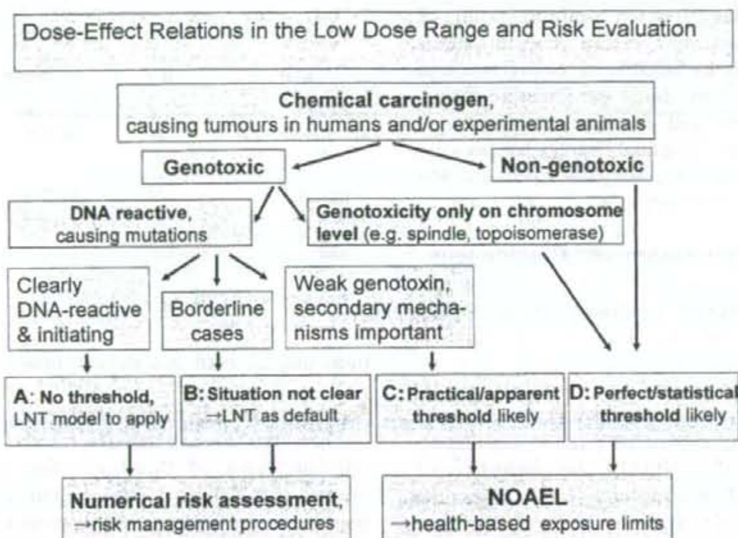

Fig. 1. Flow-chart of the SCOEL procedure to distinguish between carcinogen Groups A-D (15)

risk management regulations may be based on the ALARA principle ("as low as reasonably achievable"), technical feasibility, and other socio-political considerations.

(B) Genotoxic carcinogens, for which the existence of a threshold cannot be sufficiently supported at present. In these cases, the LNT model may be used as a default assumption, based on the scientific uncertainty.

(C) Genotoxic carcinogens with a *practical* threshold is supported by studies on mechanisms and/or toxicokinetics; health-based exposure limits may be based on an established NOAEL (no observed adverse effect level).

(D) Non-genotoxic carcinogens and non DNA-reactive carcinogens; for these compounds a true ("*perfect*") threshold is associated with a clearly founded NOAEL. The mechanisms shown by tumor promoters, spindle poisons, topoisomerase II poisons and hormones are typical examples of this category.

The flow scheme to arrive at these categories adopted by SCOEL is presented here as Fig. 1.

Application of the SCOEL Strategy for Carcinogens

Health-based OELs are derived by SCOEL for carcinogens of Groups C and D. A risk assessment is carried out by SCOEL for carcinogens of Groups A and B, whenever possible. In cases of Groups C and D, not only the mechanism of action should be well established, but also an adequate set of data is needed.

Problems may arise in considering mechanisms of genotoxicity at the chromosomal level (*e.g.* differentia-

Table 2. Results of SCOEL discussions on individual carcinogens (by 2008) and assignment to groups based on mode of action (published evaluations and evaluations under "public consultation")

Group	Description / Substances
Group A	*Non-threshold genotoxic carcinogens; for risk low-dose assessment the linear non-threshold (LNT) model appears appropriate:*
	1,3-butadiene (quantitative risk assessment performed), dimethyl sulfate, diethyl sulfate (analogy to dimethyl sulfate), hexamethyl phosphotriamide, methylene dianiline (MDA; *4,4'*-diamino-diphenyl-methane), vinyl chloride (quantitative risk assessment performed), vinyl bromide (risk assessment by analogy to vinyl chloride).
Group B	*Genotoxic carcinogens, for which the existence of a threshold cannot be sufficiently supported at present. In these cases the LNT model may be used as a default assumption, based on the scientific uncertainty:*
	acrylamide, acrylonitrile, *o*-anisidine, arsenic, benzene (provisional assignment), *2,6*-dimethylaniline (insuff. data), hexavalent chromium compounds (quantitative risk assessment performed), naphthalene, wood dust.
Group C	*Genotoxic carcinogens for which a practical threshold is supported and for which a health-based OEL is proposed:*
	dichloromethane/methylene chloride, formaldehyde, glyceryl trinitrate, lead (provisional OEL proposed), lead chromate, nickel (under discussion), pyridine, silica, trichloroethylene, vinyl acetate.
Group D	*Non-genotoxic carcinogens and/or non DNA-reactive carcinogens; for these compounds a true ("perfect") threshold is associated with a clearly founded NOAEL. A health-based OEL is proposed:* carbon tetrachloride, chloroform, nitrobenzene

tion between aneugenic and clastogenic effects; Group D) or in the differentiation of weak genotoxicants with secondary mechanisms of carcinogenesis (Group C), but progress is being made in the incorporation of mechanistic data in these instances.

Table 2 presents an overview of current results concerning specific compounds. Summary documents of the assessments by SCOEL have either been published (3,4), or are in the state of "public consultation". Examples of argumentations for key compounds are presented in the following. These compounds are also included in Table 1. The examples highlight especially the differentiation between groups B and C, which is most decisive for setting a health-based OEL.

Application of the SCOEL Procedure to Cases of Key Compounds

Case 1; Formaldehyde (Group B or C): The case of formaldehyde has been discussed very much in-depth in many EU countries (29–33). Mechanistic assessments have been published (21). Experimentally, inhaled formaldehyde produces nasal carcinomas in rats, and IARC has categorized formaldehyde as a "*Group 1*" carcinogen because the development of human nasopharyngeal carcinomas (34). In its assessment scheme SCOEL has regarded formaldehyde as a Group C carcinogen. The main arguments were that there was no straightforward evidence for a systemic genotoxic and carcinogenic effect, and that cell proliferation following chronic irritation was necessary for the tumor formation. Avoidance of irritancy would therefore lead to a health-based OEL, which was proposed at 0.2 ppm.

Case 2; Vinyl acetate (Group B or C): Vinyl acetate produces local tumors at the site of application after oral and inhalation dosing in rodents. It is instantaneously hydrolyzed at the site of first contact with the organism by ubiquitous esterases to acetic acid and formaldehyde, which is also metabolized to acetic acid. At high doses, the local genotoxic effect of formaldehyde and the cell proliferation stimulus due to acidification by acetic acid together lead to carcinogenicity. Formaldehyde and acetic acid are endogenous compounds of the C_1-metabolism *via* folic acid. If the endogenous level is not substantially exceeded, no carcinogenic effect is to be expected. This reasoning is well documented in the literature (22,23). Accordingly, SCOEL regarded vinyl acetate as a Group C carcinogen and proposed a health-based OEL of 5 ppm, which also avoids local irritancy.

Case 3; Acrylonitrile (Group B or C): Acrylonitrile is acutely toxic due to cyanide formation upon its oxidative metabolism (35). Experimentally, tumors at several target sites are observed in rodents; the assessment of risk is very much debated (36). There are arguments in favor of a threshold for experimental brain tumors, such as the absence of DNA adducts in brain, observed oxidative DNA damage in astrocytes *in vivo*, reversibility of loss in gap junction communication in exposed astrocytes, and a sublinear dose-response curve. Also, the genotoxicity *in vivo* appears not very much straightforward. However, acrylonitrile is an experimental multi-organ carcinogen (brain, spinal cord, Zymbal gland, GI tract [upon oral dosing], mammary gland). This leaves many uncertainties at present, although the existence of a threshold in the carcinogenic response appears possible. Given this uncertainty, SCOEL has regarded acrylonitrile as a Group B carcinogen, based on the present state of knowledge, with no health-based OEL assigned. The high acute toxicity of acrylonitrile and the possibility of uptake through the skin require special attention in the industrial practice.

Case 4; Acrylamide (Group B or C): Similar to acrylonitrile, acrylamide is a multi-organ carcinogen experimentally (tumors in rat brain, mammary gland and tunica vaginalis of the testes). Besides, it is highly neurotoxic. There are argumentations in favor of a threshold in carcinogenicity, but again the multiplicity of target sites and of the possible mechanisms involved renders the case very difficult to assess. Similar to recommendations of others (37,38), SCOEL has preferred to regard acrylamide as a Group B carcinogen, with no health-based OEL assigned for its carcinogenicity. However, for matters of practical handling of the compound, a value was given that can prevent neurotoxicity.

Case 5; Trichloroethylene (Group B or C): Trichloroethylene has caused renal cell carcinomas in workers exposed over several years to high peak concentrations (39,40). According to experimental investigations, a local metabolic activation *via* the glutathione-dependent pathway and renal beta-lyase is involved (39,40). Specific mutation patterns in the von Hippel-Lindau (VHL) tumor suppressor gene have been reported (39,40). An apparent pre-condition of tumor development is nephrotoxicity, for which modes of action have been published. In the "public consultation" phase, SCOEL has proposed a health-based OEL of 10 ppm, in order to avoid nephrotoxicity and thereby also nephrocarcinogenicity, categorizing trichloroethylene in Group C.

Case 6; Methylene chloride/dichloromethane (Group B or C): Methylene chloride (dichloromethane) has experimentally produced liver and lung tumors in mice, but not in rats or hamsters. Again, the compound is metabolized through an oxidative (CYP2E1 dependent) and a reductive (GSTT1-1 dependent) pathway (41). The oxidative pathway leads to formation of carbon monoxide, the reductive pathway is thought to be involved in genotoxicity (42). Recent trans-species cancer risk assessments using physiologically-based pharmacokinetics (PBPK) with a probabilistic design (43) resulted in very low theoretical

risk figures for humans: for an exposure to 100 ppm for the entire working life, the cancer risk was 4.9×10^{-5}. The large species difference in susceptibility is supported by biochemical investigations showing a difference in the amino acid sequence between the murine and human GSTT1-1 that renders the murine enzyme much more active toward methylene chloride as substrate (44). Accordingly, in the "public consultation" phase SCOEL has grouped methylene chloride in Group C, with the recommendation of an OEL of 100 ppm that would avoid a carbon monoxide load of hemoglobin (CO-Hb) higher than 3–4%.

General Conclusions

With regard to establishment of OELs for carcinogens, SCOEL has employed a strategy to distinguish between four different groups of carcinogens. For justification of a health-based OEL for a genotoxic carcinogen based on a *practical threshold*, the differentiation between Groups B and C is most important (Fig. 1). As exemplified above by six outstanding cases, the most important argument is the prerequisite of cell proliferation and chronic tissue damage at the target site for tumor development (formaldehyde, vinyl acetate, trichloroethylene). Avoidance of such conditions can justify a health-based OEL (Group C). Another argument for Group C is when large species differences between humans and tumor-susceptible animals are well supported, so that the resulting cancer risk for humans, under realistic conditions of exposure, is negligible (methylene chloride).

Again, the mode of action of the individual compound is decisive. If significant open questions or doubts remain, the default position is categorization into Group B. This is not essentially the final position, because more insights into the underlying mechanisms/modes of action may lead to a reconsideration.

The whole matter of definition of *practical thresholds* for carcinogens is under scientific discussion world-wide (13,14,23,26–28,45). But the incorporation of new principles into official regulations is a slow process, and the degree of acceptance of threshold effects differs between regulatory systems (46). Given this, it is the scientist's task to develop and promote new concepts, and to embark into a continuing discourse with stakeholders and regulatory managers.

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Review

In vivo Approaches to Study Mechanism of Action of Genotoxic Carcinogens[1]

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Genotoxic carcinogens are chemicals or factors which not only induce neoplastic lesions in animal bioassays but also test positive in genotoxicity assays *in vitro* or *in vivo*. However, it is actually difficult to discriminate genotoxic and non-genotoxic carcinogens because both assays are basically independent each other, which raises a simple query as to how much the detected genotoxic potential can consequently contribute to carcinogenicity. To clarify this critical issue, we have studied the mechanisms of action of carcinogens in transgenic rats or mice carrying reporter genes, which are expected as powerful tools for the simultaneous evaluation of both genotoxicity and carcinogenicity at the same organ level. A number of studies of genotoxic carcinogens using these transgenic rodents have revealed good correlations between genotoxicity and carcinogenicity in terms of mechanism of action. On the other hand, a known non-genotoxic carcinogen dicyclanil increased *in vivo* genotoxicity as well as oxidative DNA damage in female mice, consistently with the sex specificity of its carcinogenicity, albeit without clear evidence of direct DNA reactivity. In contrast, a genotoxic chlorinated water by-product MX failed to exert *in vivo* genotoxicity and carcinogenicity in mice. We also confirmed that such reporter gene-carrying rodents are not susceptible or resistant to carcinogenicity as compared with intact counterparts. These results thus indicate that understanding of the detailed mechanism of carcinogenic action could be crucial for more precise risk assessment, and bioassay systems using transgenic rodents carrying reporter genes would be extremely useful for that purpose.

Key words: ***in vivo*** **study, mechanism of action, genotoxic carcinogen**

Introduction

Genotoxic carcinogens are chemicals or factors which not only induce neoplastic lesions in long-term animal bioassays but also test positive in genotoxicity assays *in vitro* or *in vivo*. In this context, test chemicals are classified into 4 categories, *i.e.*, genotoxic carcinogens, non-genotoxic carcinogens, genotoxic non-carcinogens and non-genotoxic non-carcinogens. Based on this classification, genotoxic and non-genotoxic carcinogens are evaluated without and with thresholds in current risk assessment procedures, respectively (1). However, it is actually difficult to discriminate genotoxic and non-genotoxic carcinogens because each assay is carried out separately. Namely, both assays are basically independent each other, which raises a simple query as to how much the detected genotoxic potential can contribute to carcinogenicity.

In this context, several possibilities are proposed for the critical issues that genotoxic carcinogens may also have the threshold. First, if a set of genotoxicity assays for a compound carcinogenic to rodents proved to be falsely positive, the compound is no more called as a genotoxic carcinogen, indicating the existence of true threshold as a non-genotoxic carcinogen. It may be difficult to confirm the genotoxic potential found in an assay as false positive, however, false reactions in a number of genotoxicity assays obviously exist judging from some discrepancy between *in vitro* and *in vivo* assays, as well as single dose and repeated dose *in vivo* studies. Second, it is unclear as to how much the detected genotoxicities contribute to the carcinogenicity found in long-term rodent assays. This point could be important to understand organ-, species- and sex-differences of carcinogenicity. Third, it is well known that carcinogenesis process *per se* involves multi-steps such as DNA adduct formation/repair, gene mutation, apoptosis, cell proliferation and immune suppression. If there is a threshold in some of these steps, it is likely that the carcinogenic compound may have the threshold in inducing carcinogenicity. Even in the simplest hypothesis, both genotoxic and non-genotoxic or epigenetic events are required for the completion of carcinogenesis, suggesting a possible threshold determined with non-genotoxic

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events. Finally, statistical or mathematical approaches are concerned with possible practical thresholds even for true genotoxic carcinogens.

Taken together, it can be emphasized that the mechanisms of action are crucial to determine as to whether the initiation of carcinogenicity is based on the direct DNA reaction, how much the genotoxicity contributes to the carcinogenicity, or if the carcinogenicity also fits to human risks. In this review article, our data using transgenic rodent mutation models/assays are shown, and the usefulness for investigating the mechanisms of carcinogenic action is discussed.

Transgenic Rodents Carrying Reporter Genes

Recently, a detailed review on several transgenic rodent mutation assays has been reported from OECD (2), in which Muta™ Mouse, Big Blue®, *lacZ* plasmid mouse, *gpt* delta rodents, use of the λ *cII* transgene and other transgenic systems such as *supF*, *lacI* (BC-1), *rpsL* and bacteriophage ΦX174 are shown as promising models (2). Among them, the *gpt* delta mouse was established by microinjection of λEG10 phage DNA (48 kb) into the fertilised eggs of C57BL/6J mice (3). Phage λEG10 carries about 80 copies of the transgene in a head-to-tail fashion at a single site of chromosome 17 and is maintained as a homozygote (*i.e.* the mouse carries about 160 copies of λEG10 DNA per diploid genome) (4). More recently, *gpt* delta rats have been developed in Sprague-Dawley (5) and F344 (6) backgrounds. The *gpt* delta rat has approximately 10 copies of the λEG10 vector integrated at position 4q24-q31. The transgenic rat is available as a hemizygote only (5). Mutation in the *gpt* delta mouse and rat can be assessed using 6-thioguanine and Spi$^-$ selection, which respond primarily to point mutation and deletion, respectively (7).

To clarify the critical issues pointed out in the Introduction section, we have studied the mechanisms of action of carcinogens in transgenic rats or mice carrying reporter genes, which are expected to provide powerful tools for the evaluation of both genotoxicity and carcinogenicity at the same organ level.

Examples of Simultaneous Evaluation of Genotoxicity and Carcinogenicity in Transgenic Rodents

Our studies of genotoxic carcinogens such as environmental pollutants, nitrosamines and heterocyclic amines in these transgenic rodents have revealed good correlations between genotoxicity and carcinogenicity in terms of mechanism of action (6,8,10–16).

In order to cast light on carcinogen-specific molecular mechanisms underlying experimental hepatocarcinogenesis in rats, *in vivo* genotoxicity and mutation spectra of known genotoxic rat hepatocarcinogens *N*-nitrosopyrrolidine (NPYR), and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), as well as the non-genotoxic hepatocarcinogen di(2-ethylhexyl)phthalate (DEHP) and the non-carcinogen acetaminophen (APAP) were investigated in *gpt* delta transgenic rats (8). After 13-week treatment, glutathione *S*-transferase placental form (GST-P)-positive liver cell foci were significantly increased in NPYR- and IQ-treated rats. In the DEHP-treated rats, marked hepatomegaly with centrilobular hypertrophy of hepatocytes occurred, although GST-P staining was consistently negative. There were no increases in GST-P positive foci in the APAP-treated rats. Positive genotoxicity was detected in IQ- and NPYR-treated rats of which mutant frequencies in the liver DNA were approximately 35-fold and 10-fold higher, respectively, than that of non-treatment control rats. There were no increases in mutant frequencies in the DEHP- or APAP-treated rats as compared to the non-treatment control value. IQ induced mainly base substitutions leading to G:C to T:A transversions and deletions of G:C base pairs. In contrast, NPYR primarily caused specific AT to GC transitions, which are very rare in the other groups. These data provided support for the conclusion that hepatocarcinogenesis by IQ and NPYR depends on genotoxic processes and specific DNA adduct formation while DEHP exerts its influence via a non-genotoxic promotional pathway. Our data also indicate that analysis of specific *in vivo* mutational responses with transgenic animal models can provide crucial information for understanding the molecular mechanisms underlying chemical carcinogenesis (8). In fact, thymine adducts were detected at levels as much as guanine adducts in the liver of rats given NPYR (9).

To clarify the role of 8-hydroxydeoxyguanosine (8-OHdG) formation as a starting point for carcinogenesis, we examined the dose-dependence and time-course of changes of OGG1 mRNA expression, 8-OHdG levels and *in vivo* mutations in the kidneys of *gpt* delta rats given $KBrO_3$ in their drinking water for 13 weeks (6). There were no remarkable changes in OGG1 mRNA in spite of some increments being statistically significant. Increases of 8-OHdG occurred after 1 week at 500 ppm and after 13 weeks at 250 ppm. Elevation of Spi$^-$ mutant frequency suggestive of deletion mutations occurred after 9 weeks at 500 ppm although no mutations were increased before 5-weeks treatment (6). In a two-stage experiment, F344 rats were given $KBrO_3$ for 13 weeks then, after a 2-week recovery, treated with 1% nitrilotriacetate (NTA), a known kidney tumor-promoter, in the diet for 39 weeks. The incidence and multiplicity of renal preneoplastic lesions in rats given $KBrO_3$ at 500 ppm followed by NTA treatment were significantly higher than in rats treated with NTA alone. Results suggest that a certain period of time, more than

5 weeks in this experiment, might be required for 8-OHdG to cause permanent mutations. The two-step experiment shows that cells exposed to the alteration of the intranuclear status by oxidative stress including 8-OHdG formation might be able to form tumors with appropriate promotion (6).

On the other hand, dicyclanil, a mouse hepatocarcinogen showing all negative results in various genotoxicity tests increased *in vivo* genotoxicity as well as oxidative DNA damage in mice (17). Male and female *gpt* delta mice were given dicyclanil at a carcinogenic dose for 13 weeks. Significant increases in 8-OHdG levels and centrilobular hepatocyte hypertrophy were observed in the treated mice of both sexes. Bromodeoxyuridine-labeling indices and liver weights for the treated females, but not the males, were significantly higher than those for the controls. Likewise, the *gpt* mutant frequencies in the treated females were significantly elevated, GC:TA transversion mutations being predominant. The results for the transgenic mutation assays were consistent with dicyclanil carcinogenicity in terms of the sex specificity for females although it still remains unclear how much sex hormone contributes to these sex differences. Together with the early onset of 8-OHdG formation being observed 4 weeks after the treatment in the female B6C3F1 mice, a back strain of *gpt* delta mice, considering that 8-OHdG induces GC:TA transversion mutations by mispairing with A bases, it is likely that cells with high proliferation rates and a large amounts of 8-OHdG come to harbor mutations at high incidence. The results imply that examination of carcinogenic parameters concomitantly with reporter gene mutation assays is able to provide crucial information to comprehend the underlying mechanisms of so-called non-genotoxic carcinogenicity (17).

It is well documented that a chlorinated water by-product 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) is strongly mutagenic in Ames assay without metabolic activation (18) and induces carcinogenicity targeting the liver, thyroid and lung in rats (19). In contrast to dicyclanil, MX exerted neither *in vivo* genotoxicity nor carcinogenicity in mice (20). Groups of male and female *gpt* delta transgenic mice were given MX at doses of 0–100 ppm in their drinking water for 12 weeks, and then killed to assess *in vivo* genotoxicity, and cell proliferative activity using immunohistochemistry for proliferating cell nuclear antigen (20). Further groups of *gpt* delta mice were given 0 or 100 ppm MX for 78 weeks, and a full necropsy with histopathological examination of all organs was conducted to detect neoplastic lesions. The 12-week MX treatment did not result in genotoxicity in the livers or lungs or cell proliferative activity in several organs of the mice, and the 78-week treatment did not cause carcinogenicity. These findings indicate that MX is not genotoxic, mitogenic or carcinogenic in mice, and suggest that the compound might exert epigenetic actions for carcinogenicity in rats although its *in vivo* genotoxicity remains unknown in rats (20).

Approaches for Genotoxic Carcinogens

Once a certain chemical was evaluated to be positive for both genotoxicity and carcinogenicity assays, the risk of such "genotoxic carcinogen" is assessed on non-threshold basis in the current risk assessment procedure. Based on properties of non-threshold, any genotoxic carcinogens are basically prohibited when intentionally added to food like food additives, or assessed with virtually safety dose (VSD) or margin of exposure (MOE) approaches when unavoidable from environment including food (1). For "genotoxic carcinogens", it may be critical to clarify the mechanism of action. As the first step, species specificity could be determined from the weight of evidence (21,22). For example, α_{2u}-globulin-related renal carcinogenicity in male rats is no more relevant to human risk (23). If the mechanisms are more or less relevant to human risk, then contribution of genotoxicity to carcinogenicity should be evaluated. For this purpose, transgenic rodents carrying reporter genes would be very useful for judging direct, indirect or no DNA reactivity *in vivo*. Taken together with other mechanisms of action such as cell proliferation, apoptosis and immunodeficiency, the key event for carcinogenicity would tell us as to whether there is any threshold. In some cases, other transgenic rodents such as *p53*, *nrf2* or constitutive active/androstane receptor (CAR) knockout mice might be helpful to elucidate the mechanisms of action. Finally, statistical or mathematical evaluation can provide VSD or MOE even for strictly defined genotoxic carcinogens like radiation.

Conclusions

These results clearly indicate that understanding of the detailed mechanisms of carcinogenic action could be crucial for more precise risk assessment, and bioassay systems using transgenic rodents carrying reporter genes would be extremely useful for that purpose. We also confirmed that such reporter gene-carrying rodents are not susceptible or resistant to carcinogenicity (10,14,20) as compared with intact counterparts. Taken together, we propose a combined subchronic toxicity/*in vivo* genoxicity study using such transgenic rodents (Fig. 1) as a rapid and advanced bioassay to detect genotoxic carcinogens. In terms of additional approaches to detect *in vivo* genotoxic potential at organ levels, our proposing bioassay system may be more promising than a bioassay system extended from subchronic toxicity study suggested by Dr. Cohen (24).

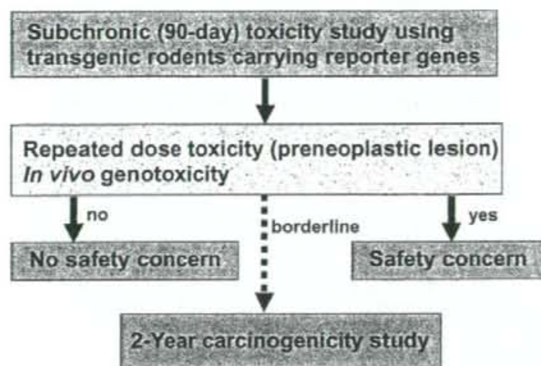

Fig. 1. Proposal of a combined subchronic toxicity/*in vivo* genoxicity study.

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Review

Possible Involvement of Adaptation Mechanisms in the Achievement of an Ineffective Dose Range for the Carcinogenicity of Genotoxic Carcinogens[1]

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Recent findings have indicated that there may be a practical threshold or an ineffective dose range for the carcinogenicity of genotoxic carcinogens. In male Fischer 344 rats given a 16-week chronic feeding administration of 0.0001–1 ppm of *N*-nitrosodiethylamine (DEN), glutathione *S*-transferase placental form (GST-P)-positive liver preneoplasias developed at 0.1 ppm or higher, but hepatic level of 8-oxoguanine (8-oxoG), an oxidative DNA damage, was not elevated even at 1 ppm. In contrast, hepatic 8-oxoG level was elevated by a single intraperitoneal administration of 0.001–100 mg/kg body weight of DEN within 6 h and remained high within 72 h, in a clear dose-dependent manner without any ineffective doses, and GST-P-positive preneoplasias correspondingly developed through the selection procedure. The 8-oxoG level was elevated also in extrahepatic organs within 6 h but returned to the normal level within 72 h. In a separate experiment, hepatic 8-oxoG level remained high even 18 weeks after 2 weekly intraperitoneal administrations of 100 mg/kg body weight of DEN. The early prolonged elevation of 8-oxoG level in target organ DNA was similarly induced by heterocyclic amines and dimethylarsinic acid in association with the down-regulation of the *Ogg1* gene encoding an 8-oxoG-specific repair enzyme. Taken together, it is suggested that adaptation mechanisms may be involved in the achievement of an ineffective dose range for the carcinogenicity of genotoxic carcinogens during their continuing exposure at sufficiently low level doses.

Key words: genotoxic carcinogen, threshold, (pre)neoplasia, 8-oxoguanine, *Ogg1* gene

Introduction

In general, the magnitude of any effects of exogenous stimuli (chemicals, microorganisms and radiation) is dependent on the exposure doses of such stimuli, and this is principally true also in the situations of carcinogenesis. There was a dogma insisting the absence of threshold levels for carcinogenic effects of carcinogens, because they were thought to be mostly mutagenic and interacting with DNA to cause irreversible genotoxicity with a strict dose dependency down to zero (1–3). Even after the general consensus about the existence of non-genotoxic carcinogens (indirectly affecting DNA and exerting carcinogenic effects through epigenetic mechanisms) and the presence of threshold levels for their carcinogenic effects (4–6), threshold levels are still believed absent for carcinogenic effects of genotoxic carcinogens (directly interacting with DNA and thereby exerting carcinogenic effects) because of their genotoxic nature itself, despite several challenging data (7–18).

A 16- and 32-week multi-laboratory study was conducted using 1145 male 20-day-old Fischer 344 rats to assess detailed dose-dependency of hepatocarcinogenic effects of a heterocyclic amine, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), featuring the lowest dietary dose of 0.001 ppm that is relevant to the human exposure level (10,11,14). As a result, MeIQx-DNA adduct was formed for all assessed doses, of which level increased according to the dose increment in the liver. Hepatic level of 8-oxoguanine (8-oxoG), an oxidative DNA injury, and glutathione *S*-transferase placental form (GST-P)-positive preneoplasias were also increased dose-dependently but only at the dose ranges of 1–100 and 10–100 ppm, respectively. It is thus apparent that ineffective dose ranges are present for the ability of MeIQx to induce oxidative DNA injury and

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putatively preneoplastic lesions. The presence of the identical ineffective dose range was confirmed also for the development of hepatocellular neoplasia for MeIQx by the long-term carcinogenicity study in rats (10). These findings are supportive for the idea that a threshold may be present even for the carcinogenic effects of genotoxic carcinogens, and indicate a concept such that the earlier an event occurs during the carcinogenic processes, the smaller an ineffective dose range is

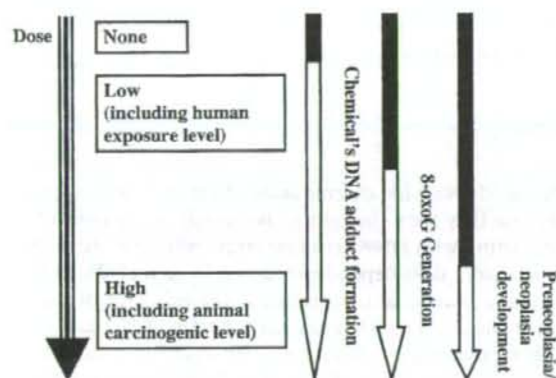

Fig. 1. The concept of the cascading ineffective dose ranges for events occurring in the carcinogenic process of genotoxic carcinogens (11,14). Closed bars indicate respective ineffective dose ranges. The earlier an event occurs during the carcinogenic processes, the smaller an ineffective dose range is. An early event such as the formation of DNA adducts may have no or smaller ineffective dose ranges compared to a late event such as the neoplasia development.

(Fig. 1) (11,14). An early event such as the formation of DNA adducts may have no or smaller ineffective dose ranges compared to a late event such as the neoplasia development. This concept looks likely, because those events occurring before the development of phenotypically apparent tumor, including carcinogen-DNA adduct formation and oxidative DNA injury generation, require additional events with genetic and/or epigenetic alteration(s) to progress into the development of morphological (pre)neoplasia. In fact, DNA adduct levels are frequently increased linearly according to the dose of carcinogens (even at the low dose range) but not strictly correlated with the eventual formation of neoplasia (19,20), and 8-oxoG is formed equally in both preneoplastic and non-preneoplastic cell populations of the target organ in some "oxidative" carcinogenic occasions (21,22).

The presence of an ineffective dose range of genotoxic carcinogens may be attributed to the biological host adaptation that would be expected in response to the low-dose (and continuous) exposure of DNA-effective agents in general (8,23,24). In the present review, some of our supportive data is demonstrated regarding this issue.

The Case of Continuous Administration of *N*-Nitrosodiethylamine (DEN)

Another multi-laboratory study was conducted using 1957 male 20-day-old Fischer 344 rats to assess detailed dose-dependency of hepatocarcinogenic effects of a nitroso-compound, DEN (11,14). In the study, DEN was continuously administered at dietary doses of 0,

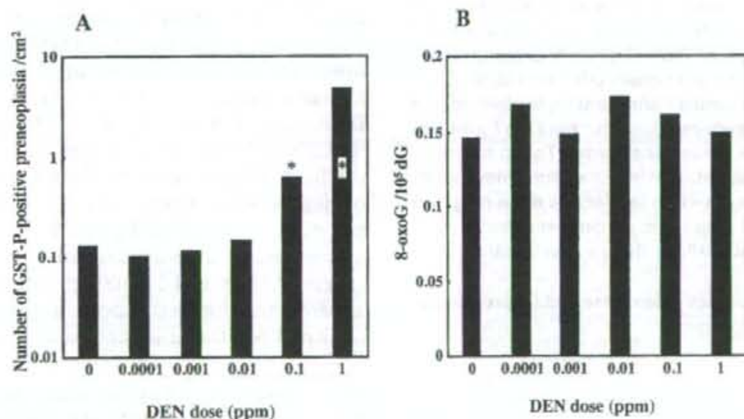

Fig. 2. Dose-relationship for the development of hepatocellular preneoplasia (A) and oxidative DNA injury (B) in rats continuously administered DEN for 16 weeks (11,14). The data of the highest dose group (10 ppm) was not obtained because of the extreme numerousness of preneoplasia. Data is demonstrated as a mean, and the ordinate in Fig. 2A is set as a logarithmic scale. Asterisks indicate that values are significantly different from the control value. Number per unit area of GST-P-positive preneoplasia was not different from the control level at the DEN dose up to 0.01 ppm, whereas the number significantly increased at the DEN dose of 0.1 ppm and further increased according to the dose increment. In contrast, 8-oxoG level was not altered by any DEN dose.