Genes and Environment, Vol. 30, No. 4 pp. 114–119 (2008)

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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(Received August 11, 2008; Revised September 29, 2008; Accepted October 1, 2008)

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

[1]Presented at the *International Symposium on Genotoxic and Carcinogenic Thresholds*, Tokyo, July 22/23, 2008

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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,

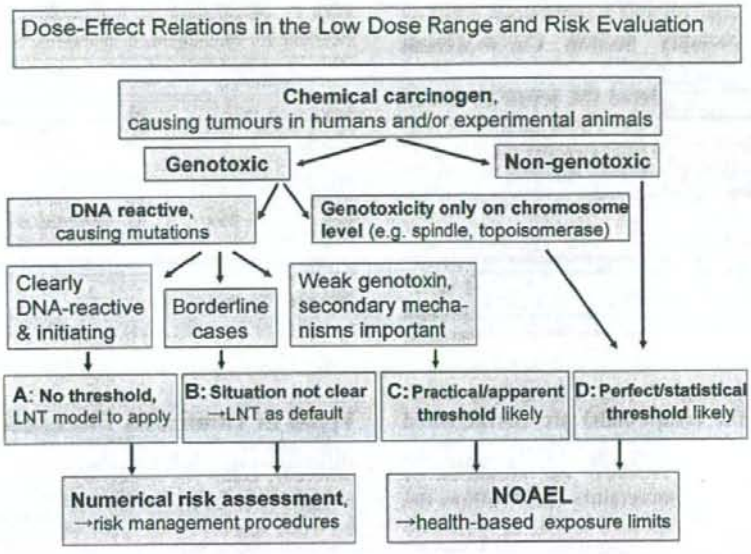

Fig. 1. Flow-chart of the SCOEL procedure to distinguish between carcinogen Groups A-D (15)

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 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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Genes and Environment, Vol. 30, No. 4 pp. 120 – 124 (2008)

Review

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

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(Received September 5, 2008; Revised October 6, 2008; Accepted October 7, 2008)

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

[1]Presented at the International Symposium on *Genetic and Carcinogenic Thresholds*, Tokyo, Japan, July 22-23, 2008.

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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).

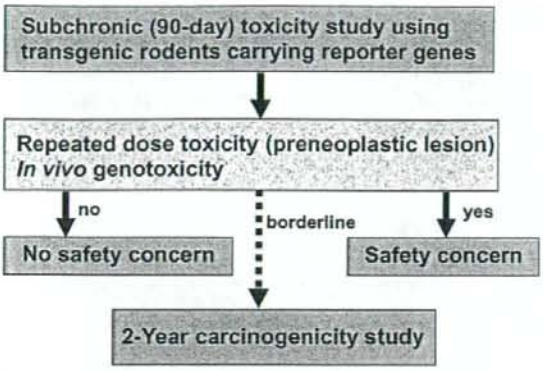

Fig. 1. Proposal of a combined subchronic toxicity/*in vivo* genoxicity study.

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Genes and Environment, Vol. 30, No. 4 pp. 125–131 (2008)

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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(Received September 1, 2008; Revised October 2, 2008; Accepted October 3, 2008)

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

[1]Most of the content of this review was presented at the International Symposium on *Genotoxic and Carcinogenic Thresholds*, held in Tokyo, Japan, on July 22 and 23, 2008.

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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) (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).

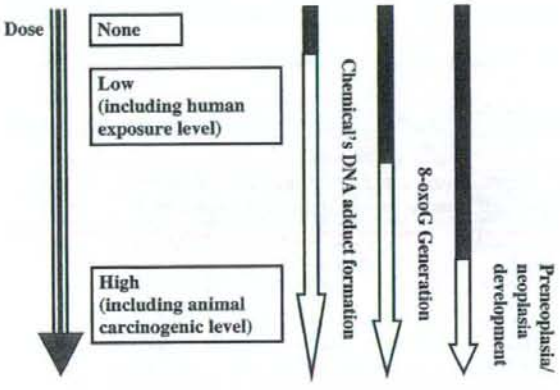

Fig. 1. The concept of the cascading ineffective dose ranges for events occurring in the carcinogenic process of genotoxic carcinogens (11,14). Closed bars indicate respective ineffective dose ranges. The earlier an event occurs during the carcinogenic processes, the smaller an ineffective dose range 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.

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,

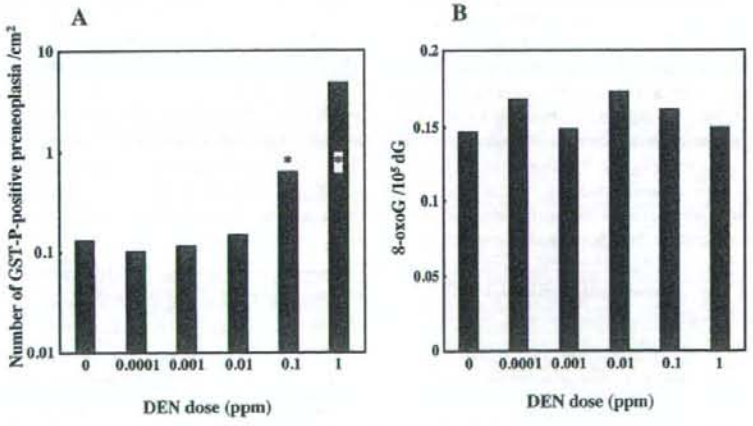

Fig. 2. Dose-relationship for the development of hepatocellular preneoplasia (A) and oxidative DNA injury (B) in rats continuously administered DEN for 16 weeks (11,14). The data of the highest dose group (10 ppm) was not obtained because of the extreme numerousness of preneoplasia. Data is demonstrated 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.

0.0001, 0.001, 0.01, 0.1, 1 and 10 ppm for 16 weeks. The lowest dietary dose of 0.0001 ppm was set because of the relevance to the human exposure level (25,26). As a result, 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 (Fig. 2A). There is thus an apparent ineffective dose range for the carcinogenicity of DEN, in well accordance with the studies in the literature (13,15–17). In contrast to the aforementioned MeIQx study, however, 8-oxoG level was not altered by any DEN dose (Fig. 2B).

The Case of Single Administration of DEN

Male Fischer 344 rats, 6 weeks old, were administered DEN at single intraperitoneal doses of 0, 0.001, 0.01, 0.1, 1, 10, 20 and 100 mg/kg body weight, and sacrificed 6, 24, 48 and 72 h thereafter, while some rats were given the selection procedure (a two-third partial hepatectomy at h 4, intraperitoneal administration of 500 mg/kg body weight of colchicine at days 1 and 3, a dietary administration of 0.02% of 2-acetylaminofluorene and a single gavage administration of 1 mL/kg body weight of carbon tetrachloride) and sacrificed at the end of week 5 (27). In the liver, 8-oxoG level significantly increased at h 6, peaked at h 24 and then gradually decreased but still high at h 72 in all DEN-treated animals (Fig. 3A). This increase of the 8-oxoG level was dose-dependent (27). This kinetics of the early change of 8-oxoG level in liver DNA is well in line with that of DEN-DNA adduct level (28–32). Similarly, number per unit volume of GST-P-positive preneoplasia significantly increased by all doses of DEN in a dose-dependent manner (Fig. 3B). Statistic analysis revealed that the dose-dependent changes of 8-oxoG level and preneoplasia number were closely correlated (27). In extrahepatic organs, 8-oxoG level also significantly increased within 6 h after the DEN administration (100 mg/kg body weight), but in contrast to the liver situation the level returned to the background level before h 72 (Fig. 4). In addition, when DEN was administered as 2 consecutive weekly intraperitoneal doses of 100 mg/kg body weight to 6-week-old male Fischer 344 rats, 8-oxoG level in liver DNA was 5.45/10^6 dG at 18 weeks after the commencement, which was still significantly higher than the control value of 0.54/10^6 dG. It is thus indicated that the 8-oxoG generation immediately after the carcinogen exposure and its maintenance at high levels within a certain period in target organ DNA is involved in the initiation mechanism of DEN. The repair system for 8-oxoG seems to be disturbed in the liver exposed to DEN, at least for a certain period, after its single (or double) administration even at low doses, while such a system well works in extrahepatic non-target organs, which can reflect the organotropic carcinogenicity of this carcinogen.

In order to corroborate the above findings, we conduced a control experiment featuring acetaminophen (APAP), an analgesic that causes hepatotoxicity by vir-

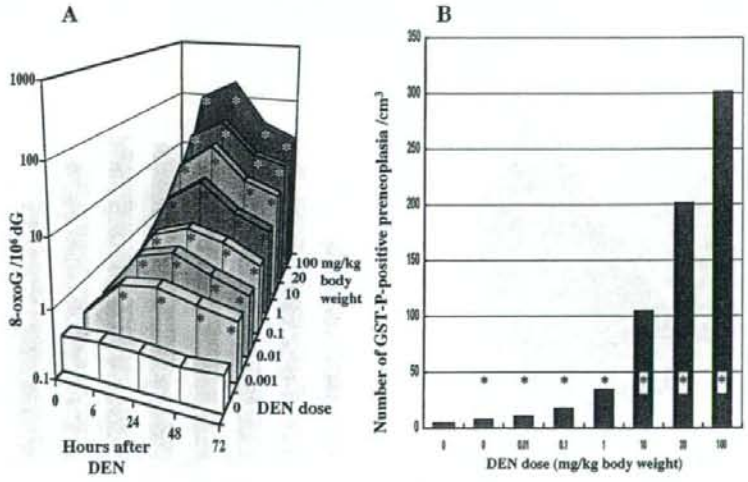

Fig. 3. Dose-relationship for the development of oxidative DNA injury within 72 h (A) and of hepatocellular preneoplasia after the selection procedure at week 5 (B) in rats given a DEN single intraperitoneal administration (27). Data is demonstrated as a mean, and the ordinate in Fig. 3A is set as a logarithmic scale. Asterisks indicate that values are significantly different from the control value. Hepatic 8-oxoG level significantly increased at h 6, peaked at h 24 and then gradually decreased but still high at h 72 in all DEN-treated animals. This increase of the 8-oxoG level was dose-dependent. Similarly, number per unit volume of GST-P-positive preneoplasia significantly increased by all doses of DEN in a dose-dependent manner.

tue of oxidative stress but not hepatocarcinogenic (33–35). Male Fischer 344 rats, 6 weeks old, were administered APAP at single intraperitoneal doses of 0, 15, 30, 60, 125, 250, 500 and 1000 mg/kg body weight, and sacrificed 6, 24, 48 and 72 h thereafter, while some rats were given the selection procedure (a dietary administration of 0.02% of 2-acetylaminofluorene and a single gavage administration of 1 mL/kg body weight of carbon tetrachloride) and sacrificed at the end of week 5. In the liver, 8-oxoG level significantly increased sometime within 48 h but returned to the background level by h 72 in animals treated with APAP at 30 mg/kg body weight or higher (Fig. 5A). This increase of the 8-oxoG level was dose-dependent. Similarly, and as expected, number per unit volume of GST-P-positive neoplasia was not altered by any doses of APAP (Fig. 5B).

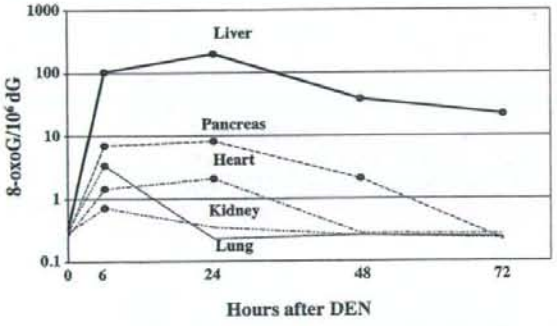

Fig. 4. Early 8-oxoG level time-course after a single intraperitoneal administration of DEN (100 mg/kg body weight) in various organs (27). Data is demonstrated as a mean, and the ordinate is set as a logarithmic scale. Closed circles indicate that values are significantly different from the control value. In extrahepatic organs, 8-oxoG level significantly increased within 6 h after the DEN administration (100 mg/kg body weight), but in contrast to the liver situation the level returned to the background level before h 72.

It is thus apparent that the prolonged maintenance of high 8-oxoG level in target organ DNA of rats exposed to DEN is not just a refection of oxidative stress but results from more complicated mechanisms including organotropic disturbance of the repair system for 8-oxoG, which is then involved in the initiation of carcinogenesis. Importantly, these phenomena regarding the single administration case of DEN lack apparent ineffective dose ranges, being different from its continuous administration case.

Early Changes for 8-oxoG Level and *Ogg1* Gene Expression after Single Administrations of Carcinogens

Male Fischer 344 rats, 6 weeks old, were administered MeIQx, 2-amino-1-methyl-6-phenylimiazo[4,5-*b*]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) or dimethylarsinic acid (DMAA) and sacrificed at h 72, when the liver and kidney were obtained and used for the assessments of 8-oxoG level and mRNA expression of the *Ogg1* gene encoding 8-hydroxyguanine DNA glycosylase, a major and specific repair enzyme for 8-oxoG (36). MeIQx, PhIP and IQ are heterocyclic amines, and MeIQx and IQ target the liver but not the kidney, while PhIP does not target the liver or kidney,

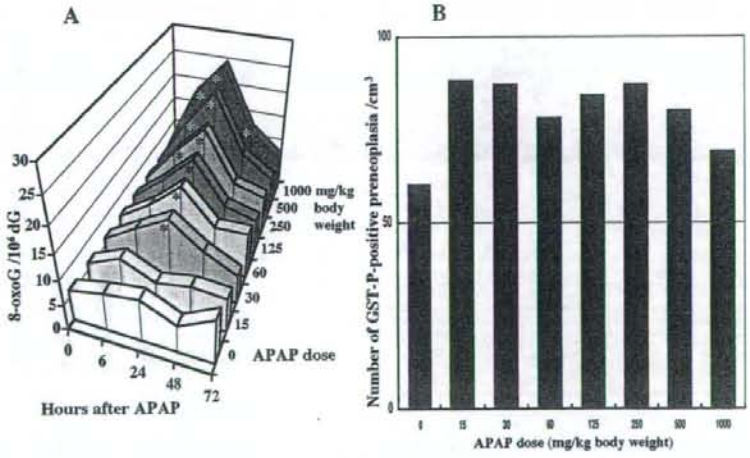

Fig. 5. Dose-relationship for the development of oxidative DNA injury within 72 h (A) and of hepatocellular preneoplasia after the selection procedure at week 5 (B) in rats given an APAP single intraperitoneal administration. Data is demonstrated as a mean. Asterisks indicate that values are significantly different from the control value. Hepatic 8-oxoG level significantly increased sometime within 48 h but returned to the background level by h 72 in animals treated with APAP at 30 mg/kg body weight or higher. This increase of the 8-oxoG level was dose-dependent. Number per unit volume of GST-P-positive neoplasia was not altered by any doses of APAP.

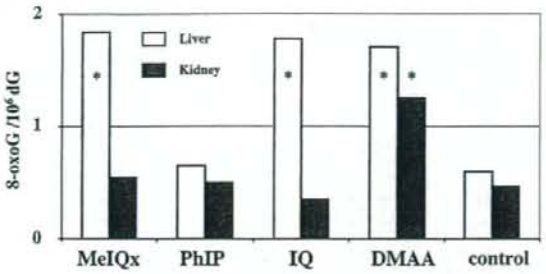

Fig. 6. Hepatic and renal 8-oxoG levels 72 h after a single administration of heterocyclic amines and DMAA. Data is demonstrated as a mean, and as indicated in the panel, open and closed bars represent data for the liver and kidney, respectively. Asterisks indicate that values are significantly different from the control value. Hepatic 8-oxoG levels in rats given MeIQx, IQ and DMAA, but not PhIP, were significantly higher than that in control animals, and renal 8-oxoG levels significantly increased in only DMAA-administered animals.

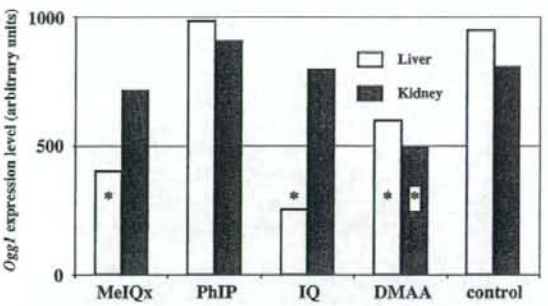

Fig. 7. Hepatic and renal *Ogg1* mRNA expression 72 h after a single administration of heterocyclic amines and DMAA. Data is demonstrated as a mean, and as indicated in the panel, open and closed bars represent data for the liver and kidney, respectively. Asterisks indicate that values are significantly different from the control value. Hepatic *Ogg1* mRNA expression was significantly down-regulated in rats given MeIQx, IQ and DMAA, but not PhIP, and renal *Ogg1* mRNA expression was significantly down-regulated in only DMAA-administered animals.

in rats (37–40). DMAA is an organic arsenic compound and targets both the liver and kidney (41–43). The heterocyclic amines were administered at single gavage doses of 0.01, 1 and 100 mg/kg body weight, whereas DMAA was administered at single intraperitoneal doses of 0.005, 0.5 and 50 mg/kg body weight. The low or middle doses of any used chemicals did not alter 8-oxoG levels or *Ogg1* mRNA expression. In the high dose cases, hepatic 8-oxoG levels in rats given MeIQx, IQ and DMAA, but not PhIP, were significantly higher than that in control animals, and renal 8-oxoG levels significantly increased in only DMAA-administered animals (Fig. 6). Conversely, hepatic *Ogg1* mRNA expression was significantly down-regulated in rats given MeIQx, IQ and DMAA, but not PhIP, and renal *Ogg1* mRNA expression was significantly down-regulated in only DMAA-administered animals (Fig. 7).

The target organ-specific down-regulation of *Ogg1* mRNA expression demonstrated here suggests that the repair system for 8-oxoG in such organs is indeed disturbed. Although DEN unfortunately was not included in this study, it can be speculated that the early prolonged high 8-oxoG level in the target organ DNA in the case of single administration of DEN may also be due to the disturbance of its repair system.

Conclusive Statements

Clear difference of effects of singly and continuously administered DEN on hepatic 8-oxoG level demonstrates the absence of an ineffective dose range in the former. Because this absence seems to result from the target organ-specific disturbance on the 8-oxoG repair system, the lack of 8-oxoG level increase in the latter case is suggested due to the recovery of the system. It is thus plausible that this recovery may be achieved as a result of adaptation caused by the continuous exposure of DEN, which thus support the aforementioned understanding that 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 risk assessment process for chemicals, one often faces difficulties in terms of the extrapolation from the data obtained in animal studies generally using high doses to the situation in humans who are usually exposed much lower doses. An issue regarding the possible presence of an ineffective dose range, or a "threshold", in the carcinogenicity of genotoxic carcinogens and its underlying mechanisms is a typical example. Waddell has recently reviewed numerous animal carcinogenicity studies in the literature by using a statistical technique and concluded that thresholds for carcinogenesis are logically indicated present for virtually all of his reviewing carcinogens, that the range of most of such carcinogenic thresholds is within the range of blood or serum concentrations for therapeutic drugs in current use, that the mere presence of DNA adducts in a tissue does not predict the tumor formation, and that hormesis applies to carcinogenesis (44). In this Waddell's review, the threshold for carcinogenicity of aflatoxins is estimated from the animal study data to be $10^{15.5}$ molecules/kg body weight/day, which is amazingly close to that estimated from the epidemiologically obtained human data ($10^{15.7}$ molecules/kg body weight/day) (44). It is thus really critical to investigate what happens in animals administered test chemicals at sufficiently low doses, and such efforts as introduced and referred in this article can be useful to overcome the above-mentioned difficulties concerning the extrapola-

tion from the animal data to the human situation in the risk assessment process.

Taken together, it is suggested that adaptation mechanisms may be involved in the achievement of an ineffective dose range for carcinogenicity of genotoxic carcinogens during their continuous exposure at sufficiently low level doses. This is another issue to be settled in order to make consensus regarding the concept of ineffective dose ranges, or "thresholds", for carcinogenicity of genotoxic carcinogens.

Acknowledgments: The authors would like to express their gratitude to numerous people participating in 2 multi-laboratory studies for detailed dose-response of the MeIQx and DEN carcinogenicities, who are represented by Drs. Masao Hirose (Food Safety Commission, Chiyoda, Tokyo, Japan), Katsumi Imaida (Kagawa University, Takamatsu, Kagawa, Japan), Tomoyuki Shirai (Nagoya City University, Nagoya, Aichi, Japan), Masae Tatematsu (Aichi Cancer Center Research Institute, Nagoya, Aichi, Japan), Hiroyuki Tsuda (Nagoya City University, Nagoya, Aichi, Japan) and Keiji Wakabayashi (National Cancer Center Research Institute, Chu'oh, Tokyo, Japan). We thank Dr. Hiroshi Kasai and his group (University of Occupational and Environmental Health, Kita-Kyushu, Fukuoka, Japan) for their cooperation and Dr. Akihiko Maekawa (National Institute of Technology and Evaluation, Shibuya, Tokyo, Japan) for his generous supervision.

Original and published works introduced herein were supported in part by Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Corporation (JST), Grants-in-Aids 06280119 and 09253105 for Scientific Research on Priority Areas, Cancer Research, from the Ministry of Education, Science, Sports and Culture of Japan, Research Grant of the Princess Takamatsu Cancer Research Fund of Japan (01-23308), Grant of the Foundation for Promotion of Cancer Research of Japan, Grant-in-Aid for Cancer Research 12-16 from the Ministry of Health, Labour and Welfare of Japan, and Grant R01 CA82506 from the National Institutes of Health of USA.

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Genes and Environment, Vol. 30, No. 4 pp. 132–138 (2008)

Review

Thresholds in Genotoxicity and Carcinogenicity: Urinary Bladder Carcinogenesis[1]

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(Received August 6, 2008; Revised October 27, 2008; Accepted October 31, 2008)

Cancer is due to multiple alterations to DNA. Chemicals can increase the cancer risk by directly damaging DNA (DNA reactivity) or by increasing cell proliferation (DNA replications), increasing the number of opportunities for spontaneous DNA damage. Genotoxicity is a more comprehensive term than DNA reactivity. Many of the mechanisms of genotoxicity, such as clastogenicity, inhibition of DNA repair, or damage to the mitotic apparatus, produce DNA damage indirectly. These non-DNA reactive mechanisms involve interactions with proteins and mechanistically are threshold phenomena. 2-Acetylaminofluorene (AAF) is DNA reactive. Its dose response for urinary bladder DNA adduct formation is linear, whereas the tumor response is non-linear. Non-linearity is at the dose at which increased cell proliferation occurs, related to the threshold phenomenon of cytotoxicity. Non-linearity for DNA reactive carcinogens can also be produced by changes in metabolic processes of activation and/or deactivation due to saturable kinetics. Arsenic produces bladder cancer with a non-linear dose response in animal models and humans. Genotoxicity of arsenic occurs secondarily to indirect mechanisms, not DNA reactivity, it has a non-linear dose response, and the genotoxic mechanism appears to have a threshold, occurring only at doses in excess of toxic concentrations. Numerous non-genotoxic agents have been identified as bladder carcinogens in rodent models, most acting by inducing cytotoxicity with regenerative proliferation. Cytotoxicity can be produced by formation of urinary solids or by urinary reactive chemicals. Urinary solids are a defined threshold phenomenon based on the physical-chemical property of solubility. Likewise, chemical induction of cytotoxicity is a known threshold phenomenon. Non-genotoxic chemicals have a threshold dose response with respect to carcinogenesis, as do most genotoxic agents. DNA reactive chemicals have a non-linear dose response.

Key words: sodium saccharin, calculi, arsenic, acetylaminofluorene, genotoxicity

Numerous chemicals have been identified as causing cancer in humans. To avoid the release of additional chemical carcinogens into the environment, numerous screening tests have been developed to try to identify such chemicals so that they can be avoided. Nevertheless, numerous chemical carcinogens remain in the environment, although most are present at extremely low levels of exposure. The question arises as to whether any of these low levels of exposure pose an actual cancer risk to humans, or is there a level of exposure below which there is no risk, a so-called threshold.

In this presentation I will first present a theoretical framework of carcinogenesis on which to evaluate this question, and then present several examples illustrating various aspects of this question. I am focusing on urinary bladder carcinogens since there is considerable information regarding chemicals that can induce bladder cancer either in experimental models or in humans (1). Numerous specific chemicals and mixtures have been identified as human bladder carcinogens, and much is known about their metabolic activation processes, kinetics, and dynamics. In addition, numerous extensive epidemiologic investigations have been performed to investigate various aspects of chemical carcinogenesis with respect to the urinary bladder.

Chemicals have been known to be responsible for the induction of bladder cancer since the first original observation by Rehn in 1895 of an association between exposure of workers in the aniline dye industry in Germany and the development of bladder cancer (1,2). Research into this problem eventually led to the identification of aromatic amines as the chemicals responsible for carcinogenicity (2). Extensive research into the metabolic processes of aromatic amines led to the development of the reactive electrophile theory of carcinogenesis proposed by the Millers (3). Numerous other chemicals have been identified as human bladder carcinogens, including phosphoramide mustards and ar-

[1]Presented at the International Symposium on *Genotoxic and Carcinogenic Thresholds*, Tokyo, Japan, July 22-23, 2008.

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senic, but the most important causative agent for bladder cancer in developed countries is cigarette smoking (1). Cigarette smoke contains large quantities of various aromatic amines, but especially 4-aminobiphenyl. In addition to various chemical exposures, infectious diseases have also been identified as causative agents for bladder cancer, including schistosomiasis and bacterial cystitis. Radiation exposure to the pelvis has also been identified as causative for bladder cancer (1,2).

After years of extensive research, much is known about the process of carcinogenesis (4,5). Fundamentally, we now know cancer is due to genetic alterations, usually occurring in somatic cells. It has also become clear that more than one genetic alteration is required for cancer to arise. All of the genetic alterations necessary for cancer development must occur in a single cell. These genetic mistakes become permanent only if they occur during DNA replication. Furthermore, although DNA replication is incredibly precise, spontaneous errors occur each time DNA replicates. Based on these assumptions regarding carcinogenesis, there are ultimately only two ways by which an agent, chemical or otherwise, can increase the risk of cancer: 1) increase the rate of DNA damage directly; or 2) increase the number of DNA replications (4,5). The chemicals which directly damage DNA have been referred to as DNA reactive or genotoxic. I will use the more restrictive term, DNA reactive. Agents which induce cancer by increasing DNA replication are referred to as non-DNA reactive or non-genotoxic, and act by increasing cell proliferation. Most DNA reactive agents also increase cell proliferation at high exposure levels, but usually not at lower exposure levels (6).

An essential aspect of the carcinogenesis paradigm is that all of the necessary errors in the DNA must occur in the stem cell population of a tissue (4). This is the population of cells that provides for replacement and repair of tissues when there is injury or toxicity. Under normal circumstances, when a tissue stem cell divides, it produces a replacement stem cell and a cell that is committed for eventual differentiation. Differentiation is a cell death process. As differentiated cells die, they are replaced by this proliferative process of normal stem cells. When there is toxicity or injury to a tissue, resulting in an overall loss of stem cells in the population, normal stem cells replicate into two stem cells until the stem cell population is replaced, at which time normal differentiation processes can again occur. Every time DNA replication occurs within a normal stem cell, there is a rare probability that a mistake can occur in one of the genes that is necessary for the ultimate development of cancer. As the mistakes that are necessary for cancer accumulate in the cell, during each of the subsequent DNA replications there is a probability of additional mistakes occurring until the ultimate number of mistakes have occurred that lead to the production of a malignant cell. Malignant cells replicate, frequently more rapidly than their normal counterpart, but most malignant stem cells still have the capability of undergoing differentiation, although to a more limited extent than their normal counterpart.

The "spontaneous" errors that occur during DNA replication are due to the numerous endogenous chemical alterations that occur on the DNA on a regular basis, including oxidative damage, exocyclic adduct formation, and many other events which occur numerous times daily on the DNA (7). Although most of these chemical alterations of the DNA that occur endogenously are repaired, occasionally one leads to a permanent error in the DNA. DNA reactive carcinogens increase the amount of DNA damage per replication by forming additional adducts on the DNA. Again, although most of these are repaired, some lead to permanent errors in the DNA. By increasing the number of DNA replications, the actual number of mistakes can increase even though the rate per DNA replication does not change.

For chemicals that produce urinary tract cancer, they can either be DNA reactive or non-DNA reactive (8). As indicated above, the first identified bladder carcinogens were the aromatic amines which are DNA reactive. If the bladder carcinogen is not DNA reactive, then it increases bladder cancer risk by increasing cell proliferation. This can be due to direct mitogenesis (one example is known, propoxur in the rat), but more commonly the chemical increases cell proliferation by inducing toxicity with consequent regenerative proliferation. Toxicity can be induced in the urinary bladder either by the formation of urinary solids (precipitate, crystals, or calculi), or the chemical or a metabolite can be cytotoxic directly to the urothelial cells. There is some evidence suggesting that in the animal model extreme abnormalities of urinary composition can lead to toxicity and consequent regeneration, such as extremes in urinary pH or volume, although it is unclear whether this is accompanied by other alterations, including chemical cytotoxicity or formation of solids.

Since toxicity is usually, if not always, a threshold event, chemicals that induce cancer by toxicity and regenerative proliferation consequently have a threshold level (9,10). This is most obvious for agents related to the formation of urinary solids (11,12). Solids will only form if the solubility of the substance is exceeded. This is a physical property of a chemical in the specific solvent milieu, in this case, urine. For such agents, there is a clear threshold response, so that if administration of the chemical is at a dose that is high enough to produce the urinary solids, there is toxicity and ultimately tumor formation. If the dose is insufficient, below the threshold, to produce urinary tract solids, then there is