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Review

in vivo Approaches to Identify Mutations and *in vitro* Research to Reveal Underlying Mechanisms of Genotoxic Thresholds

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In regulatory toxicology, it is assumed that genotoxic carcinogens, which induce cancer through genotoxic mechanisms, have no threshold for their action. However, humans possess a number of defense mechanisms against DNA damaging agents, which may reduce the genotoxic and cancer risk at low doses to the spontaneous levels. The defense mechanisms may constitute practical thresholds for genotoxic carcinogens. In fact, accumulating evidence with rodent carcinogenicity and genotoxicity assays suggest that some genotoxic compounds clearly exhibit threshold-like dose responses *in vivo*. These results challenge the paradigm that cancer risk induced by genotoxic compounds at high doses can be linearly extrapolated into low doses where people are exposed in daily life (linear non-threshold model). Here, we discuss two issues regarding the practical thresholds for genotoxic carcinogens. The first issue is how to define ''genotoxicity'' of chemicals. There are a number of genotoxicity assays *in vitro* and *in vivo*. Therefore, it is unclear what genotoxicity assay(s) should be employed to define whether the compound is genotoxic or not. The second issue is possible mechanisms underlying the practical thresholds. In particular, we emphasize the importance of DNA repair and translesion DNA synthesis as the underlying mechanisms of the practical thresholds. Finally, we discuss issues associated with low dose exposure to genotoxic carcinogens, i.e., risk assessment of exposure to multiple genotoxic chemicals.

Key words: practical thresholds, genotoxic carcinogens, non-genotoxic carcinogens, DNA repair, translesion DNA synthesis

Introduction

Humans are exposed to a variety of chemicals that may induce damage in DNA. Although the damage may be repaired by multiple defense mechanisms against DNA damaging agents before DNA replication occurs, DNA that possesses modified bases or missing bases may be used as template for DNA replication. Such damaged DNA replication results in genetic alterations such as mutations and chromosome aberrations (1). It is widely accepted that cancer is a result of multiple genetic alterations in important genes such as those involved in maintenance of genome integrity, e.g., *p53*, or cell proliferation, e.g., *ras* (2–5). Therefore, risk assessment of genotoxicity of chemicals is critically important to protect humans from environmental carcinogens. In 1980s, a large number of chemicals have been examined for the carcinogenicity and genotoxicity at the National Toxicology Program in the United States (6). These chemicals are commercially important and may represent significant environmental and occupational hazards by virtue of their high volume of production and release to the environment. Although most of rodent carcinogens were expected to be positive in the bacterial mutation assay (Ames assay) and have structural alert to interact with DNA, about 40% of rodent carcinogens were negative in the genotoxicity assays and had no structural alerts to interact with DNA (6,7). Therefore, the term ''non-genotoxic carcinogens'' was coined to define the carcinogens that do not exhibit genotoxicity and have no structural alerts to interact with DNA (8–10). Carcinogens that were positive in genotoxicity assays were referred to as ''genotoxic carcinogens''. The former included carcinogens that may promote cancer via cell toxicity, cell proliferation, epigenetics or hormonal effects (9). Because these ''non-genotoxic carcinogens'' are neither supposed to interact with DNA nor induce mutations, they were expected to possess ''thresholds'' for their action as other toxic agents (8). No-observed-adverse-effect levels (NOAEL)

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can be set by chronic rodent bioassays and acceptable daily intake (ADI) is calculated for the non-genotoxic carcinogens (11). If the dose used in daily life is below the ADI, non-genotoxic carcinogens may be allowed to be used in the society. In contrast, genotoxic carcinogens are believed to have no thresholds because mutation is a stochastic event and even single molecules may induce mutations and cancer in humans (12). Hence, if candidate chemicals for pesticides, food additives or veterinary medicines are carcinogenic in rodents and genotoxicity is involved in the cancer mechanisms, no ADI can be set and the chemicals are not allowed to go to the markets. As shown in this special issue and other publications, however, the paradigm that genotoxic carcinogens have no thresholds has been challenged by experimentally (13). In fact, it seems plausible that at least some genotoxic carcinogens have ''practical threshold'' for their action (14–16). The term ''practical thresholds'' is defined as the doses below which no mutations are detected (12,17). In addition, self defense mechanisms such as detoxication and DNA repair may constitute the practical thresholds for genotoxicity (18).

In this report, we first discuss how we should identify genotoxicity of chemicals. As written in the next section, there are a number of genotoxicity assays *in vitro* and *in vivo*. The endpoints are diverged such as DNA damage, point mutations or chromosome aberrations. Ames test is the most widely accepted indicator for genotoxicity but has also some limitations, e.g., prokaryotes versus eukaryotes. We discuss the importance of *in vivo* gene mutations assays in rats and mice in terms of examination of possible involvement of genotoxicity in carcinogenesis in rodents (19). We then discuss possible mechanisms underlying genotoxic thresholds. In particular, we focus on DNA repair and translesion DNA synthesis (TLS) (20). Finally, we discuss other issues related to risk of genotoxic carcinogens at low doses where humans are actually exposed to the chemicals in daily life.

Pivotal Roles of *in vivo* Transgenic Gene Mutation Assays to Make a Distinction between Genotoxic and Non-genotoxic Carcinogens

Currently, there are more than 100 genotoxicity assays so far published. The organisms used for the assays include phage, bacteria, yeast, plants, insects, fish, fungi and rodents so on. In addition, cultured mammalian or human cells are frequently used for the assays. Therefore, it is not uncommon that one chemical exhibits different test results depending on the assays used, e.g., positive in one assay but negative in another assay. Of the various genotoxicity assays, bacterial mutation assays with *Salmonella typhimurium* and *Escherichia coli*, either chromosome aberration assays or gene mutation assays with cultured mammalian cells, and rodent micronucleus or chromosome aberration assays are regarded as a standard battery of genotoxicity assays (21). However, it is still not easy to define whether the chemical is genotoxic or not because each assay has its own merits and limitations to evaluate the genotoxic effects in human genome. The bacterial mutation assays could indicate the chemical or its metabolites have potential to interact with DNA and induce mutations or not. However, the prokaryotes lack the metabolism of mammals such as P450 drug metabolizing enzymes (22). The *in vitro* metabolism by S9 enzymes prepared from drug-induced rat liver does not necessarily represent the *in vivo* metabolism. Urethane and procarbazine are such examples (23,24). They are negative in Ames test but are positive in *in vivo* genotoxicity assays. Cultured mammalian genotoxicity assays have merits to detect chemicals that interact with proteins essential for chromosome segregation such as tubulines or topoisomerases (21). However, chromosome aberration assays *in vitro* give high percentage of false positive results partly because the aberrations are often induced associated with cellular toxicity (25). Rodent micronucleus assays could identify genotoxic agents *in vivo* and have low percentage of false positives. Nevertheless, the target organs for the micronucleus assays are bone marrow or peripheral blood cells. Thus, negative in the assays does not mean that the chemical dose not induce mutations in other organs such as liver. Diethylnitrosamine and 2,4-diaminotolune are such examples. These chemicals are negative in mouse micronucleus assays but are positive in gene mutation assays in the liver. In addition, they are hepatocarcinogens in rodents. A possible reason for the negative results in the micronucleus assays in the bone marrow is that the active metabolites generated in the liver are short lived and do not reach the bone marrow (26,27). Genotoxicity assays *in vivo* such as micronucleus assays are usually conducted in mice while two-year cancer bioassays are conducted in rats mainly. In fact, rats and mice are not the same and exhibit different sensitivity to a number of carcinogens. The most represent example is aflatoxin B1, which induces liver cancer in rats and humans but not in mice (28,29). Therefore, it is desirable to examine the genotoxicity in target organs for carcinogenicity in rodents used for two-year cancer bioassays.

Transgenic rodent mutation assays are *in vivo* genotoxicity assays that detect mutations in any organs of rodents. Transgenic mutation assays with *gpt* delta rats and mice are one of them and unlike other transgenic tests they allow to identify point mutations such as base substitutions and frameshift and deletion mutations by *gpt* selection and Spi^- selection, respectively (19). Initially, *gpt* delta mice have been established by microinjection of lambda EG10 DNA carrying reporter genes

for the *gpt* and Spi$^-$ selections into fertilized eggs of C57BL6/J mice (30). The mice carry about 80 lambda EG10 DNA in each of the chromosome 17, which results in high packaging efficiency (31). Mutation can be identified in any organs of mice. Actually, mutations have been analyzed with *gpt* delta mice in liver, stomach, lung, skin, colon, kidney, spleen and testis, induced by a variety of chemicals and radiation (32). Umemura *et al.* employed *gpt* delta mice to characterize the *in vivo* genotoxicity of dicyclanyl, an insect growth regulator (33). The compound is carcinogenic in the liver of female mice while the compound was negative in any of standard genotoxicity assays described above plus comet assay *in vivo*. Thus, dicyclanil was regarded as a non-genotoxic carcinogen. However, upon 13 weeks feeding of diets containing 0.15% dicyclanil, *gpt* mutant frequency was increased about four times in the liver of female mice. No induction of mutations in the male mice. This gender-specific induction of mutation seems to be female-specific cell proliferation in the liver. Umemura *et al.* have demonstrated that dicyclanil induces oxidative DNA damage in the liver of both male and female mice but enhances cell proliferation only in the female. The oxidative damage in DNA, i.e., 8-oxoguanine in DNA, appears to be induced during metabolism of dicyclanil in the liver. This work highlights the importance to examine mutations in a target organ of rodents used for cancer bioassay. It is worth examining whether dicyclanil induces mutations and cancer at much lower doses because it may be a genotoxic carcinogen.

Although mice are widely used for cancer bioassay, there are a number of chemicals that display carcinogenicity in rats but not in mice (28). Therefore, *gpt* delta rats have been established with the same reporter gene for mutations. Hayashi *et al.* have introduced lambda EG10 DNA into fertilized eggs of Sprague-Dawley (S.D.) rats and established *gpt* delta rats (34). The rats exhibit dose-dependent positive mutagenic responses in the liver when they are exposed to benzo[*a*]pyrene. Later, S.D. *gpt* delta rats were backcrossed to Fischer 344 (F-344) because F-344 rats are used for two-year cancer bioassay more often than S.D. rats (35). F-344 *gpt* delta rats are employed to examine the standard protocol of transgenic rodent gene mutation assays proposed for OECD guideline TG488, i.e., 28 days treatments plus 3 days expression time (36–38). In conclusion, when the chemical is carcinogenic in rodents and the chemical should be classified into a genotoxic or non-genotoxic carcinogen, the chemical should be examined with the standard battery genotoxicity assays first. In particular, results of Ames assay gives helpful information whether the chemical (or its metabolites) has potential to interact with DNA and induce mutations at least *in vitro*. However, if the results of the standard assays do not provide sufficient evidence to account for the mechanism(s) of carcinogenicity of the chemical, *gpt* delta transgenic rodent mutation assays may be useful. Parallel examination of carcinogenicity and genotoxicity of chemicals in target organs in F-344 rats and F-344 *gpt* delta rats, respectively, may provide crucial evidence whether the chemicals induce carcinogenicity via mutagenic effects or not.

Possible Mechanisms Underlying Practical Thresholds for Genotoxic Carcinogens

Accumulating evidence suggest that several genotoxic carcinogens exhibit threshold-like dose responses in carcinogenicity and *in vivo* genotoxicity assays (15,16,39,40). In fact, we have examined no obvious genotoxic effects of 2-amino-3,8-dimethylimidazo [4,5-*f*]-quinoxaline (MeIQx) in the liver of *gpt* delta mice (17). The mice were fed a diet containing 300, 30 or 3 ppm of MeIQx for 12 weeks and *gpt* mutant frequency (MF) was determined in the liver. Although treatments of mice with high doses of MeIQx, i.e., 300 and 30 ppm, clearly enhanced *gpt* MF, the low dose treatment at 3 ppm did not enhanced *gpt* MF significantly. DNA sequence analyses of the *gpt* mutants indicated that G:C to T:A was the most frequently induced mutation by MeIQx. Specific mutation frequency of G:C to T:A was significantly enhanced at dose of 300 or 30 ppm of MeIQx. However, no increase in the specific mutation frequency was observed at 3 ppm. From the results, 3 ppm was proposed as the practical threshold for genotoxicity of MeIQx in the liver of mice.

If practical thresholds exist for at least some genotoxic carcinogens, what will the underlying mechanisms be? An obvious possibility is that self-defense mechanisms such as low-molecular-weight scavengers, detoxication metabolism, DNA repair and TLS (18). The scavengers such as antioxidants alleviates the toxicity of reactive oxygen species and detoxication enzymes such as glutathione S-transferase mediate conjugation reactions, which enhance hydrophilicity of toxic chemicals and promote the excretion. DNA repair fixes damaged DNA by a multiple mechanisms such as removal of damaged or mismatched bases in DNA or rejoining broken DNA strands. TLS is a short DNA synthesis across DNA lesion (Fig. 1) (20). If error-free TLS occurs, the mechanism will contribute to tolerance against mutagenic and carcinogenic effects of chemicals. However, if error-prone TLS occurs, it will enhance mutation frequency and initiate carcinogenesis.

Here, we show dose-response curves of mutagenicity of L-cystein, dopamine hydrochloride, phenazine methosulfate (PMS) and L-penicillamine in strains *S. typhimurium* TA1535 and its derivative YG3206 (Fig. 2). The strain YG3206 is the same as TA1535 but lacks the nth_{ST} and nei_{ST} genes encoding endonuclease III and

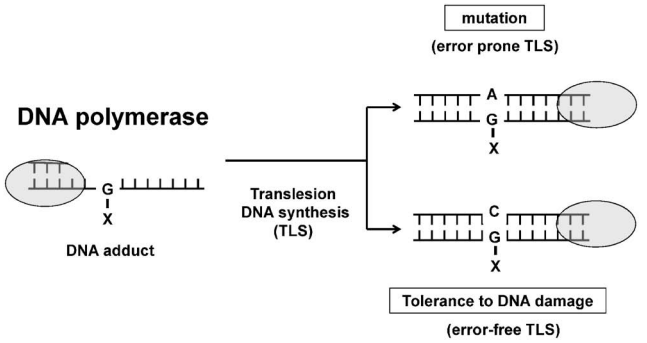

Fig. 1. Translesion DNA synthesis plays a pivotal role in induction and suppression of mutations.

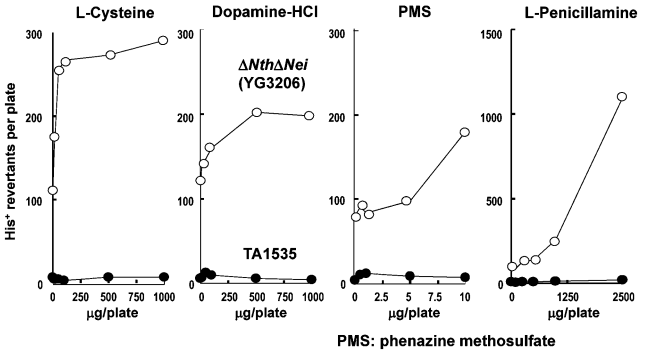

Fig. 2. Endonuclease III and VIII (Nth and Nei) are constituents of practical thresholds. Closed and open circles show the number of His^+ revertants per plate of the parent strain TA1535 and the *nth/nei* deletion derivative YG3206 lacking endonuclease III and VIII.

VIII, respectively, involved in repair of oxidized pyrimidines in DNA (41). Interestingly, the mutagenicity of the chemicals was clearly identified with strain YG3206 but not with strain TA1535. The results suggest that mutagenic oxidized pyrimidines are induced in DNA by the treatments with the chemicals but the mutagenic lesions are repaired effectively by the concerned actions of endonuclease III and endonuclease VIII. The results also raise the possibility that endonuclease III and VIII may be constituents of practical thresholds for the mutagenicity of chemicals that induce oxidized pyrimidines in DNA. Since strain YG3206 exhibited much higher spontaneous mutations compared to strain TA1535, the endonucleases may protect the genome from endogenous mutagens as well as exogenous ones. Previously, we reported that other DNA repair enzymes, i.e., O^6-methylguanine DNA methyltransferases (MGMTs) encoded by ada_{ST} and ogt_{ST} in strain TA1535, severely reduced the mutagenicity of alkylating agents (42). Mutagenicity of MNNG, ENNG, PNNG, BNNG and MMS was clearly identified with strain YG7108 lacking the ada_{ST} and ogt_{ST} genes even at very low doses but they were weakly mutagenic or not mutagenic at low doses with the parent strain TA1535. Another repair-defective derivative of strain TA1535, i.e., strain YG3001, clearly detects the mutagenicity of potassium bromated but strain TA1535 did not (43). The strain YG3001 lacks the $mutM_{ST}$ gene encoding 8-oxo-guanine DNA glycosylase involved in repair of oxidized purines in DNA. Collectively, these DNA repair enzymes protect the genome effectively from various DNA damaging agents and thus may contribute to generate practical thresholds for genotoxicity of chemicals. Endonuclease III, MGMT and 8-oxo-guanine DNA glycosylase have counterparts in humans (1). Therefore, it is interesting to examine how these counterparts constitute practical thresholds for genotoxicity in humans.

S. typhimurium and *E. coli* are both gram negative bacteria and their genome sequences are more than 80% similar. However, *S. typhimurium* LT2 and the derivatives including Ames tester strains exhibit much lower ability to induce mutations when they are exposed to various DNA damaging agents (44,45). This is probably because the $umuDC_{ST}$ gene of *S. typhimurium* has weak TLS activity although it is not experimentally demonstrated. In 1970's when Ames assay was being developed, it was demonstrated that strain TA1535 had no ability to detect mutagenicity of aflatoxin B1 even in the presence of S9 activation and furylfuramide (AF2) in the absence of S9 (46). Therefore, plasmid pKM101 was introduced to strain TA1535 and TA1538 to enhance the sensitivity. Later, it was revealed that plasmid pKM101 carried the *mucAB* genes encoding DNA polymerase R1, which is an error-prone TLS DNA polymerase (47,48). The pKM101-bearing derivatives of strain TA1535 and TA1538 are strain TA100 and strain T98, respectively, which are members of current standard Ames tester strains. In general, introduction of pKM101 enhances mutability of the host strains. However, several chemicals such as acetaldehyde oxime, 6-mercaptopurine, and 1,3-butadiene are reported to be negative or weakly positive in strain TA100 but positive in strain TA1535 (49). Humans possess more than 14 TLS DNA polymerases (50). It is a future theme whether and how these TLS polymerases affect the mutagenicity of chemicals at low doses and modulate the threshold levels of genotoxic agents.

Future Directions

Humans are exposed to a number of chemicals in daily life. Therefore, an important question is whether exposure to multiple chemicals has synergistic or additional (geno)toxic effects on humans. If genotoxicity of chemicals have no thresholds for the action, the exposure to multiple genotoxic agents should have additional effects. In fact, Ohta demonstrated that addition of six mutagenic heterocyclic amines at very low doses, each of which doesn't induce detectable mutations, give

positive mutagenic results in *S. typhimurium* when they are combined (51). The results suggest that chemicals induce mutations even below the practical threshold levels when they are combined. However, if genotoxic compounds are fractionated and administrated below the practical threshold levels, repeated exposure does not induce mutations. Gocke *et al.* (16) compared the genotoxicity *in vivo* (*lacZ* mutation in bone marrow, liver and GI tracts) between values of fractionated doses of ethyl methanesulfonate (EMS) (28 times 12.5 mg/kg) and a single dose of EMS (350 mg/kg). Although single dose gave positive mutagenic effects on the three organs, the fractionated treatments did not induce mutations in any of the organs. It suggests that EMS has a threshold for the mutagenic action *in vivo* and 12.5 mg/kg is below the threshold level.

In summary, we discussed how we evaluate the genotoxicity of chemicals and possible mechanisms underlying the practical thresholds of genotoxic chemicals. To define whether the chemical carcinogen exerts the carcinogenicity via genotoxicity, *in vivo* mutation assays such as *gpt* delta transgenic mice and rats may be informative. The assay allows to examine genotoxicity in target organs of rodents used for two-year cancer bioassay. We emphasized the importance of DNA repair and TLS as mechanisms for the practical thresholds for genotoxic chemicals. However, further work is needed to experimentally how and to what extent DNA repair and TLS affect the threshold levels of genotoxic chemicals in humans. In this regard, genetically modified mice and human cells that lack specific DNA repair or TLS functions will be useful for the purposes.

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