by *Helicobacter pylori* infection, and *ALDH2*1/2*2* are independently associated with increase of gastric cancer risk in Japanese alcoholic patients. Combinations of CAG and *ALDH2*1/2*2* showed greater risks of gastric carcinoma (*OR* = 39.2 for severe CAG plus *ALDH2*1/2*2*, CAG negative plus *ALDH2*1/2*1* was used as a reference) [11]. Zhang et al. also reported that another polymorphism of *ALDH2* found in Europeans, *ALDH2* Ex1 + 82A > *G*, is associated with alcohol-related increase in gastric cancer risk in Poland, although biochemical properties of this new polymorphism are not well understood [13]. These data suggest that acetaldehyde plays crucial roles in the development of stomach cancers.

Acetaldehyde is thought to be a tumor initiator because of its mutagenic and DNA-damaging properties [14–17]. Acetaldehyde forms several DNA adducts such as N^2-ethyl-2′-deoxyguanosine (N^2-Et-dG), α-*S*- and α-*R*-methyl-γ-hydroxy-1, N^2-propano-2′-deoxyguanosine (α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG) [18–20]. Aside from these stable DNA adducts, reaction of acetaldehyde results in the formation of an unstable and more abundant DNA adduct, N^2-ethylidene-dG (N^2-Eti-dG). Wang et al. showed that N^2-Eti-dG in human liver DNA is relatively stable and that the presence of this adduct could be confirmed by detection of N^2-Et-dG after reduction of DNA during isolation and enzymatic hydrolysis [21]. Because N^2-Eti-dG is the most abundant DNA adduct among acetaldehyde-derived adducts, it would be the sensitive biomarker of acetaldehyde exposure. In this study, we analyzed N^2-Eti-dG in stomach DNA of *Aldh2*-knockout mice that were exposed to alcohol to determine the effects of alcohol consumption and *Aldh2* genotype on the level of DNA damage in the stomach.

2. Materials and methods

2.1. Animal treatment

10–11-week old male *ALDH2*-knockout mice, which had been backcrossed C57BL6, were obtained from the Department of Environmental Health, University of Occupational and Environmental Health, Japan. The mice were used in conformity with the regulation of the committee on animal experiments of Saga University, Japan. The genotype of *ALDH2* was determined by polymerase chain reaction according to the method of Kitagawa et al. using genomic DNA from their ears and lungs [22].

The mice were fed 20% ethanol solution and standard hard feed CR-LPF (348 kcal/100 g) (Charles River Japan, Yokohama, Japan) for 5 weeks. They were then killed and stomach tissue specimens were collected, frozen in liquid nitrogen and stored at −80 °C until they were analyzed.

2.2. DNA isolation from mice stomach

For quantification of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), mouse stomach DNA was extracted and purified by using Gentra® puregene™ tissue kit (QIAGEN). The protocol was performed basically as per manufacture's instructions except that desferroxamine (final concentration: 0.1 mM) was added to all solutions to avoid formation of oxidative adducts during the purification step.

For quantification of N^2-Eti-dG, DNA was isolated as described previously [23]. Gentra Puregen tissue kit was used. The procedure was basically as per manufacture's instructions except for adding $NaBH_3CN$ to all solutions (final concentration: 100 mM). After the purification step, DNA was dissolved in 10 mM Tris–HCl/5 mM EDTA buffer (pH 7.0), extracted with chloroform and precipitated with ethanol.

2.3. DNA adduct standards and their stable isotope

8-oxo-dG was purchased from Sigma–Aldrich Japan, Tokyo, Japan. [U-$^{15}N_5$] 8-oxo-dG was kindly supplied by Dr. Shibutani at SUNY Stony Brook, USA. N^2-Et-dG and its [U-$^{15}N_5$]-labeled standard was synthesized as described previously [18].

2.4. DNA digestion

20 μg aliquots of DNA were digested into their constituent 2′-deoxyribonucleoside-3′-monophosphate units by the addition of 15 μl of 17 mM citrate plus 8 mM $CaCl_2$ buffer that contained micrococcal nuclease (22.5 U) and spleen phosphodiesterase (0.075 U) plus internal standards. The solutions were mixed and incubated for 3 h at 37 °C, after which alkaline phosphatase (1 U), 10 μl of 0.5 M Tris–HCl (pH 8.5), 5 μl of 20 mM $ZnSO_4$ and 67 μl of distilled water were added and incubated for a further 3 h at 37 °C. The digested sample was extracted twice with methanol. The methanol fractions were evaporated to dryness, resuspended in 50 μl of distilled water and subjected to liquid chromatography tandem mass spectrometry (LC/MS/MS).

2.5. Instrument

LC/MS/MS analyses were performed using a Shimadzu LC system (Shimadzu, Kyoto, Japan) interfaced with a Quattro Ultima triple stage quadrupole MS (Waters-Micromass, Manchester, UK). The LC column was eluted over a gradient that began at a ratio of 5% methanol to 95% water and was changed to 40% methanol over a period of 30 min, changed to 80% methanol from 30 to 35 min and finally returned to the original starting conditions, 5:95, for the remaining 11 min. The total run time was 46 min. Sample injection volumes of 20 μl each were separated on a Shim-pack XR-ODS column (3.0 mm × 75 mm, 2.2 μm) and eluted at a flow rate of 0.2 ml/min. Mass spectral analyses were carried out in positive ion mode with nitrogen as the nebulizing gas. The ion source temperature was 130 °C, the desolvation gas temperature was 380 °C and the cone voltage was operated at a constant 35 V. Nitrogen gas was also used as the desolvation gas (700 l/h) and cone gas (35 l/h) and argon was used to provide a collision cell pressure of 1.5×10^{-3} mbar. Positive ions were acquired in multiple reaction monitoring (MRM) mode. The MRM transitions monitored were as follows: [$^{15}N_5$]8-oxo-dG, *m/z* 288 → 172; 8-oxo-dG, *m/z* 283 → 167; [$^{15}N_5$] N^2-Et-dG, *m/z* 301 → 185 and N^2-Et-dG, *m/z* 295.5 → 179.9. The amount of each adduct was quantified by the ratio of the peak area of the target adducts to that of its stable isotope. Quanlynx (version 4.0) software (Waters-Micromass) was used to create standard curves and to calculate adduct concentrations. The amount of deoxyguanosine was monitored at 254 nm by a Shimadzu SPD-10A UV-Visible detector that was in place before the tandem MS.

3. Results

The mice were fed with water or 20% ethanol and standard hard feed for 5 weeks. Feed intake slightly dropped in the 20% ethanol group, but there was no significant difference amongst the *Aldh2* genotypes. The average ethanol intake in the case of the 20% ethanol group was not significantly different between *Aldh2* genotypes (~23 g/day/kg body wt). Reduction in body weight was observed predominantly in *Aldh2*−/− mice as described in our previous report [24]. After 5 weeks of feeding with water and 20% ethanol, the mice were killed and their stomach DNA was extracted and purified to detect DNA adduct level.

Firstly, we analyzed the level of acetaldehyde-inducible stable DNA adducts, N^2-Et-dG. However, it was not detected in any stomach DNA samples for both alcohol-treated and non-treated mice for any *Aldh2* genotype. Next, to determine acetaldehyde-derived major DNA adduct, N^2-Eti-dG in DNA, we purified stomach DNA using several reagents containing strong reducing agent, $NaBH_3CN$. During the purification procedure, it was expected that N^2-Eti-dG would be converted to stable N^2-Et-dG (Fig. 1). The average N^2-Eti-dG level in DNA from untreated mice was 3.1 ± 2.3 adducts/10^7 bases in *Aldh2*+/+ mice, 2.0 ± 0.6 in *Aldh2*+/− mice, and 2.2 ± 0.4 in *Aldh2*−/− mice, respectively. On the other hand, in 20% ethanol-treated mice, significant N^2-Eti-dG level increase was observed. The average N^2-Eti-dG level in DNA from 20% ethanol-treated mice was 4.8 ± 2.6 adducts/10^7 bases in *Aldh2*+/+ mice, 7.9 ± 1.1 adducts/10^7 bases in *Aldh2*+/− mice, and 48.6 ± 12.0 adducts/10^7 bases in *Aldh2*−/− mice, respectively (Fig. 2A). This data indicated that *Aldh2* genotype has an effect on gastric N^2-Eti-dG level. On the other hand, DNA adduct induced by oxidative stress, 8-oxo-dG, was detected in all samples, but neither alcohol-dependent nor *Aldh2* genotype-dependent change was observed (Fig. 2B).

4. Discussion

Several epidemiological evidences suggested that alcohol consumption is a risk factor of gastric cancer and genotype of *ALDH2* is considered to play an important role in increasing the risk. One animal carcinogenicity study supported the epidemiological observation. Male and female Sprague–Dawley rats fed on 10% ethanol, started at 39 weeks of age and continued until their spontaneous death, showed significantly increased frequency of forestomach

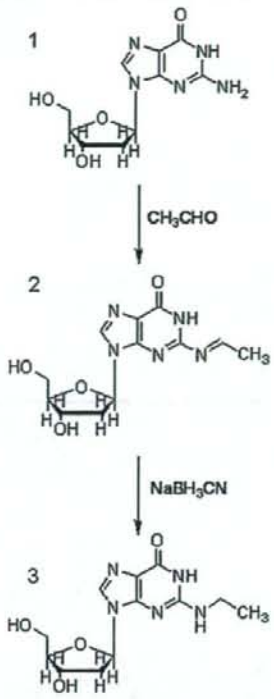

Fig. 1. Formation of acetaldehyde-dG adducts: (1) 2′-deoxyguanosine; (2) N^2-ethylidene-2′-deoxyguanosine (N^2-Eti-dG); (3) N^2-ethyl-2′-deoxyguanosine (N^2-Et-dG) N^2-Eti-dG was easily reduced with $NaBH_3CN$.

acanthoma and carcinoma [25]. However, little other experimental evidence, which support the relationship between *ALDH2* genotype and gastric cancer risk, was available. In this study, we clearly showed that ethanol significantly induced N^2-Eti-dG DNA adducts in mouse stomach, and formation of the DNA adduct was *Aldh2* genotype-dependent. This result is consistent with the report by Ogawa et al. which showed *Aldh2* genotype-dependent incorporation of radioactivity in stomach DNA of mice administered ^3H- or ^{14}C-labeled ethanol [26].

Several pathways are considered for generation of and exposure to acetaldehyde in stomach. A portion of consumed ethanol is absorbed through stomach mucosa. ADH1C is the major ADH expressed in stomach mucosa, and a portion of absorbed ethanol should be metabolized to acetaldehyde on site [9,27]. Ethanol, absorbed from digestive organs, is distributed throughout the body and the majority of it is metabolized in liver where most of the ADHs are expressed (ADH1A, ADH1B, ADH1C, ADH2, ADH3, ADH4 and ADH5) [9], and produced acetaldehyde is distributed throughout the body via circulation, and the stomach may be exposed to acetaldehyde via circulatory fluids. Another important pathway is via saliva. Ethanol contained in saliva is metabolized to acetaldehyde by oral flora [28], and the upper digestive tract, including the stomach, is continuously expose to saliva with high concentration of acetaldehyde.

The concentration of acetaldehyde in saliva after ingesting ethanol is much higher than in blood, and the acetaldehyde concentration in saliva is significantly higher in *ALDH2* deficient individuals than in normal individuals [29,30]. Alcohol challenge test of 0.6 g ethanol/kg body weight of healthy human volunteers resulted in peak acetaldehyde concentration in blood and saliva of 5 and 53 μM, respectively in *ALDH2*1/*1* homozygotes and 25 and 76 μM, respectively in *ALDH2*1/*2* heterozygotes [29]. Taking this into consideration, in this study, the stomach is considered to be exposed to higher concentration of acetaldehyde in *Aldh2*(−/−) mice than *Aldh2*(+/+) mice via both fluid and saliva pathways.

Human stomach mucosa expresses ALDH1, ALDH2 and ALDH3. The most abundant one is ALDH3, however, its K_m value is thought to be several orders of magnitude higher than that of ALDH2 [26,31]. Klyosov et al. reported that the K_m value of human ALDH1, ALDH2 and hamster ALDH3 were 180 μM, 200 nM and 3.6 mM, respectively when acetaldehyde was used as a substrate [32]. Considering realistic blood and salivary acetaldehyde concentration, ALDH3 may not work well in the stomach, and ALDH2 as well as ALDH1 will con-

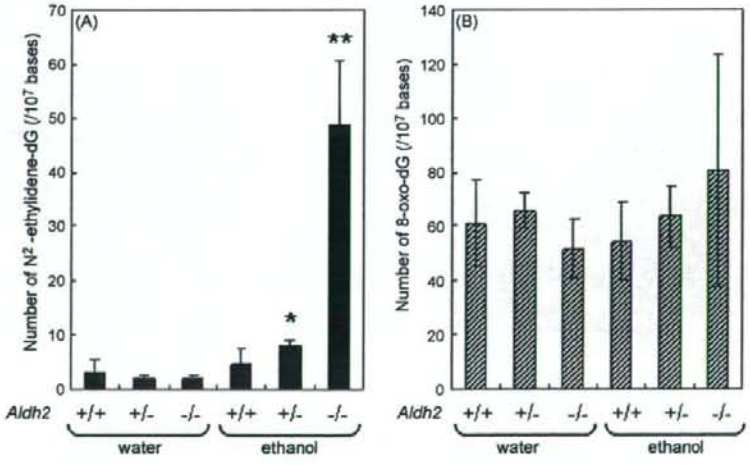

Fig. 2. DNA adduct levels in control and alcohol-treated mice having different *Aldh2* genotypes. Mice were fed with water (*Aldh2*+/+: $n=5$, (+/−): $n=6$, (−/−): $n=5$) or 20% ethanol (*Aldh2*+/+: $n=6$, (+/−): $n=5$, (−/−): $n=4$) for 5 weeks. Stomach DNA samples were purified with $NaBH_3CN$ for the determination of N^2-Eti-dG or without $NaBH_3CN$ for the determination of 8-oxo-dG. Unstable N^2-Eti-dG was reduced to stable N^2-Et-dG under the presence of $NaBH_3CN$. The level of N^2-Eti-dG was detected as N^2-Et-dG by LC/MS/MS. (A) Shows the levels of N^2-Eti-dG in mice stomach DNA and (B) shows the level of 8-oxo-dG in mice stomach. The error bars represent the standard deviation. (*) Significantly increased from water control (+/−); (**) significantly increased from water control (−/−) or ethanol-treated *Aldh2*+/+ mice ($p<0.01$).

tribute to the clearance of acetaldehyde in stomach mucosa. In this situation again, high concentration of acetaldehyde should remain longer in the stomach in *Aldh2*(−/−) mice than in *Aldh2*(+/+) mice.

In conclusion, our data clearly showed that alcohol drinking caused DNA damage in the stomach, and the risk was *ALDH2* genotype-dependent. This strongly supports the epidemiological observations which suggest alcohol drinking and *ALDH2* deficiency are risk factors of stomach cancers.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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WADAI 話題

遺伝毒性物質に閾値はあるのか？

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1 はじめに

食品の安全性に対して多くの国民が関心を寄せている今日，残留農薬や食品添加物等の食品中に含まれる微量の化学物質の安全性が問題となっている．多くの化学物質の毒性は，健康リスクを評価する場合，理論的，実証的研究から，これ以下であれば健康影響がみられないレベル，すなわち閾値がある用量反応モデルが用いられてきた．これにより1日摂取許容量(acceptable daily intake；ADI)を定めることができる．しかしながら，その化学物質の発がん性が問題となり，さらに遺伝毒性が認められるとやっかいである．他の毒性と異なり遺伝毒性には閾値がないとされているため，摂取量をゼロにしない限り健康リスクもゼロにならないとの論理からADIを設定することができない．ここに遺伝毒性発がん物質のリスク管理の問題点がある．

2 遺伝毒性とは？

遺伝毒性(genotoxicity)は遺伝子の本体であるDNAや染色体に対する毒性である．その定義は曖昧かつ広義であるが，一般には「DNAや染色体の構造的，もしくは量的変化を引き起こす性質」をいう．別の言葉として変異原性(mutagenicity)があるが，こちらは遺伝毒性に比べて狭義であり，主としてDNAや染色体に対する損傷の結果として生じる突然変異等の誘発能を示す(図1)．変異原性が最終的な遺伝的影響を示すものであり，それ以外の遺伝毒性はDNAや染色体が何らかの影響を受けたことによる一過性の変化であることが多い．遺伝毒性は他の毒性と異なり，それ自体の毒性の実態をつかむことができない．肝毒性，神経毒性，発がん性などは症状や病変として我々の体で認識できるが，遺伝毒性自体の症状や病変はない．

遺伝毒性はその結果として，がんや遺伝性疾患を引き起こす．したがって遺伝毒性とは，それら疾患を引き起こすポテンシャルの1つであり，その有無は遺伝毒性試験によって認識される．図1に一般的な遺伝毒性試験を示す．遺伝子DNAはバクテリアからほ乳類まで共通する生命の設計図であり，様々な動物種を用いた試験法が開発されている．また，そのエンドポイントはDNAの損傷，染色体の構造的，もしくは数的変化，遺伝子突然変異等，多岐にわたる．このなかで代表的な試験法としてはエームス試験，染色体異常試験，小核試験(*in vivo*)が挙げられる．これら試験は医薬品を初めとする多くの化学物質の安全性を評価する上で必須の試験として義務づけられている．

遺伝毒性試験は一般的に，遺伝毒性ハザードの有無を検出する定性的試験法であり，その結果は「陽性」もしくは「陰性」として判定される．しかしながら，毒性には本来，量的相関性

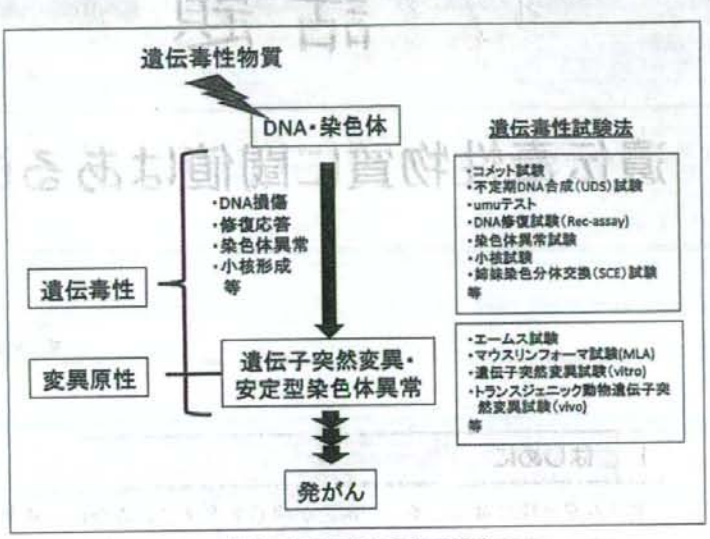

図1　遺伝毒性とその試験法

があることが常識であり，遺伝毒性のような一義的に陽性，陰性を決定することの方が特殊といえる．したがって，遺伝毒性の閾値問題はこの結果の定性的評価法に端を発するといえよう．最近になってトランスジェニック動物の開発等によって遺伝子突然変異試験等の定量的試験法が *in vivo* で実用可能となった．また，遺伝子突然変異はがんを引き起こす直接要因であることから，その試験結果は発がん性遺伝毒性物質評価の重要なエビデンスにもなりうる．

3　遺伝毒性発がん物質，非遺伝毒性発がん物質

遺伝毒性試験の目的の1つは，化学物質等の発がん可能性を調査するためのスクリーニングである．しかし，遺伝毒性試験で陽性となったからといっても必ずしも発がん性があるとは限らない．遺伝毒性試験結果とげっ歯類発がん性試験結果の相関性は，試験系によっても異なるが60～80%程度である．スクリーニングとしての目的上，できるだけ多くの発がん可能性を検出することが求められるため，感度の高い試験法が開発・利用されてきたが，それでも一部の発がん性物質に関しては陰性を示す．これらが非遺伝毒性発がん物質である．がんは遺伝子の病気であり，必ず遺伝的な変化を伴うと考えられるが，これらの物質は自然に生じたがん原細胞の増殖の亢進などを通じてがんの形成を助けるものと考えられる．ホルモン作用を持つ化学物質の一部などがこれに相当する．

遺伝毒性物質にはベンツピレン，アフラトキシンB1，N-ニトロソ化合物，アルキル化剤などの強力な発がん物質が含まれる．これら化学物質はDNAに直接作用し，切断，架橋，付加体の形成，脱塩基，酸化損傷，アルキル化等を引き起こし，その結果，高い確率で突然変異を引き起こす．一方，遺伝毒性試験で陽性であっても直接DNAに作用しないものもある．チュブリンの重合阻害剤であるコルヒチンは細胞分裂装置に影響を与え，染色体異常を引き起こす．また，DNA修復阻害，アポトーシス抑制，細胞周期停止などを引き起こす化学物質も遺伝毒性試験で陽性を示すことがある．

これら，化学物質のターゲットはDNAではなくタンパク質であり，非DNA損傷性遺伝毒

性物質と定義することができる．*In vitro* 遺伝毒性試験は陽性反応を示しやすく，その毒性メカニズムが不明であることもある．強い細胞毒性，高浸透圧，沈殿の生成，非生理的 pH などは非特異的な影響により陽性反応を示すこともあるので注意を要する．通常，1つの遺伝毒性試験の結果から遺伝毒性の有無を判定することは困難であり，複数の試験結果から試験条件や反応の程度などを考慮して判定することが多い．遺伝毒性試験で陰性を示すもの，また陽性を示しても非 DNA 損傷性であるものを総称して非遺伝毒性物質と呼ぶこともある．

4 遺伝毒性物質に閾値はあるのか？

遺伝毒性物質が DNA と反応し遺伝子突然変異をもたらす．突然変異は確率論的(stochastic)事象であり 0 になることはない．また，たった1つの遺伝子突然変異でも，その変異ががん遺伝子，がん抑制遺伝子などの細胞のがん化に重要な遺伝子に生じた場合，1つのがん原細胞が生じ，それだけで発がんに至ることがある．したがって，この発がんの確率も 0 にはならず，理論的に遺伝毒性発がん物質に閾値を設定することはできない．

一方，タンパク質に作用する非遺伝毒性発がん物質に関してはどうであろうか？ 1つの細胞中には遺伝子は多くても 2 コピーしか存在しないのに対して，タンパク質分子は数多く存在する．高濃度の化学物質が多くのタンパク質と作用すれば発がんに至る影響が表れるかもしれないが，少数であれば影響はないことは容易に想像できる．このようなことから非遺伝毒性発がん物質に関しては理論的に閾値が設定できる．また，幾つかの実験により非遺伝毒性発がん物質の閾値の存在は証明されており，多くの専門家はこの問題に関して異論はない．問題は遺伝毒性発がん物質の閾値である．

遺伝毒性発がん物質の発がん性，遺伝毒性，DNA 付加体の形成に閾値が存在するかどうかの検討が多くの研究者によって動物実験等によってなされている．アフラトキシン B1 やベンツピレンを動物に投与した場合，肝 DNA 付加体の形成は用量相関性を示す．DNA 付加体の検出は質量分析機の進歩により通常，人が曝露するレベルより 2 桁低いレベルの検出まで可能となっており，極低用量でも用量相関性が観察される．DNA 付加体の形成は化学反応であり，DNA と反応する化学物質が存在する限り形成を否定できないため閾値がないとするのが一般的である．

生物学的反応である遺伝毒性の閾値の存在の証明には，動物に投与する遺伝毒性物質の用量を段階的に下げて無作用量が存在するかどうかを，様々な遺伝毒性のエンドポイントで検出する方法がとられている．遺伝子突然変異試験の場合，無作用量とは自然誘発突然変異レベルを示す．このような実験は千～十万倍の用量域で行うため，用量を対数換算して表示することがしばしば見られる．図 2 は $y=ax+b$ の用量相関性を示す反応の 2 つのグラフを示す．これは閾値なしモデルであるが，対数表示だと閾値があるようにみえる．これは錯覚であり，このような図から閾値を論じるべきではない．同様に，低用量域ではその増加量が極くわずかで有意差がないため閾値とみなすとする論理もあるが，これも正しくない．それは試験の検出力が乏しく，自然突然変異の変動が大きいため統計的に有意にならないだけである．先に述べたように遺伝毒性は遺伝毒性試験によって認識される．すべての試験には検出限界があり，用量を段階的に下げて，無作用量が存在するかどうかをみるというストラテジーは，閾値よりもむしろ検出感度をみるに過ぎない．そもそも(閾値の)存在を，非検出をもって証明することが論理的に無理があるように思われる．

一方，極低用量域では高用量域からの一義的外挿では説明できない生物学的反応が効率的に働くため閾値を設定できるとの説もある．ここでの生物学的反応とは DNA 修復，代謝反応，

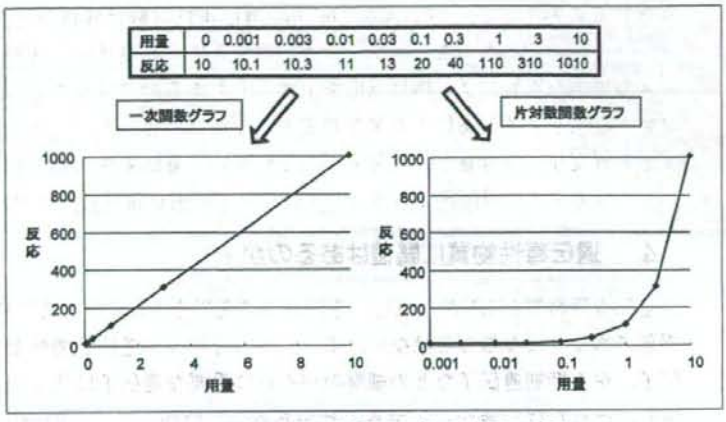

図2 閾値なし用量相関モデルのグラフ表示

スカベンジャーなどの防御機構が考えられる．しかしながら，このような防御機構は遺伝毒性の発生確率の低減化には寄与するが，閾値を作る根拠にはならない．DNA 付加体の除去には塩基除去修復機構が働き，これは一般にエラー非発生型の修復機構であるが，10^{-6} 以下の発生頻度でエラーが起き，突然変異を引き起こす．同様に，化学物質の無毒化に働く薬物代謝や，スカベンジャーも 100% の効率で働くという保証はない．

このような科学的・理論的解釈では閾値を設定できないが，現実的には極低用量の遺伝毒性反応は，自然に起きる反応と区別をすることが困難であり，閾値と見なしてもいいのではないかという考え方もある．一般に遺伝子突然変異試験の自然突然変異頻度は 10^{-6} 程度であり，一定のばらつきを持つ．この原因として酸化ストレス，老化等の内的要因や，環境中に極微量存在する試験物質以外の化学物質や放射線，紫外線などの影響が考えられる．これにより，自然に起きる遺伝毒性反応内に収まるようなレベルを「現実的閾値(practical もしくは pragmatic threshold)」とするものである．しかし，これは閾値とは別の問題である．現実的閾値の考えはある種の妥協であり，専門家の中でもこの考えに合意はできていない．したがって，発がん物質が遺伝毒性試験陽性，特にそれが発がん標的組織であれば，その発がん性に閾値を設定することはできず，ADI のような安全量を設定することはできない．

5 遺伝毒性発がん物質のリスク管理

それでは，遺伝毒性発がん物質に閾値が設定できなければそのリスク管理はできないのであろうか？ 米国においては 1958 年に「発がん性の可能性がある化学物質はいかなる低用量でも安全とみなすことはできない」という，いわゆるデラニー条項により，動物に対して発がん性を示す農薬が残留する加工食品の販売が禁止され，その後，適用範囲が着色料，動物用薬品，飼料に拡大された．しかしながら，このゼロリスク思想は現実的には多くの矛盾点があった．主な矛盾点としては，①分析技術の進歩により，微量な化学物質も検出可能となり，検出限界である安全レベルがどんどん低くなってしまうこと．②発がん性の有無だけが強調されているため，他の毒性が低くて，安全性の高い化合物ができても，わずかの発がん性のため代替できないこと．③人工化学物質のみを対象としているため，天然由来の発がん物質は無視されていること．④動物実験の発がん性試験は，必ずしも人に対する発がん性と一致しないこと，などが挙げられる．

これらのことから，1996年「食品品質保護法」の制定とともにデラニー条項は廃止された．

閾値を設定しゼロリスクを追求するのに対して，「発がん可能性がある化学物質が十分に低濃度であれば，その発がん可能性は極めて小さくなり，その程度が社会的に許容できるリスクレベルであれば実質的に安全と見なし得る」とのリスク管理の方法もある．この量を実質安全性量(virtually safety dose；VSD)といい，そのリスクレベルを「無視しうる(negligible)」，もしくは「許容できる(acceptable)」リスクとする．ここでの許容できるリスクとしてのがんの生涯リスクレベルは一般的に百万分の1(10^{-6})が採用されている．10^{-6}の生涯リスクとは日本の人口(10^8)と，平均寿命(80)から計算すると($10^8\times1/80\times10^{-6}=1.25$)1年間に1.25人のがんによる死者が増えることを意味する．がんは今や先進諸国では死亡原因の1位であり，我が国においても年間約35万人が，がんで死亡していることを考慮すると1.25人の増加は社会的に許容できるといえよう．VSDは一般にげっ歯類を用いた発がん試験で得られた半数がん誘発用量(TD 50)からマルチステージモデル，もしくは直線外挿により得られる(図3)．このような発がん化学物質を生涯発がんリスクレベルで評価し，管理に用いる手法は，現在，水道水や大気中に含まれる汚染物質の新しい環境基準値の設定に用いられている．

Cheesemanらは約500種類の発がん化学物質に関する動物実験でのTD 50からVSDを算出し，その算定曝露分布の結果から，ほとんどの発がん化学物質については0.5 μg/kg(0.5 ppb)以下の食事中濃度で百万分の1のがん生涯リスクよりも低くなることを示した．[1] 1人の1日食事量を3 kg(固形食品1.5 kg，飲料1.5 kg)とし，その化学物質が全食事にムラなく入っていると仮定すると，1日曝露量は1.5 μg/人と計算できる．つまり，大部分の化学物質については1日の摂取量が1.5 μg/人以下であれば，たとえそれが発がん物質であっても実質的な健康危害はほとんどないだろうとすることができる．このような包括的な閾値を「毒性学的懸念の閾値(threshold of toxicological concern；TTC)」という．TTCは化学構造を考慮すればその毒性が分かっていないものも含め，多くの化学物質に適用できる．我が国では食品衛生法に基づき残留農薬のポジティブリスト制が導入されたが，ここでは残留基準値が設定されていない農薬に関しては一律基準値として0.01 ppmが設定された．この値もTTC(1.5 μg/人)に基づくものであり，個々の農畜産物の1日摂取量は米を除いて150 gを超えることがないという国民栄養調査から計算されている(1.5/150＝0.01)．TTCは既に米国FDAがプラスチック容器から溶出する化学物質(間接添加物)のリスク管理に用いており，またJECFA(FAO/WHO合同食品添加物専門家委員会)は食品に添加する香料物質に適用している．

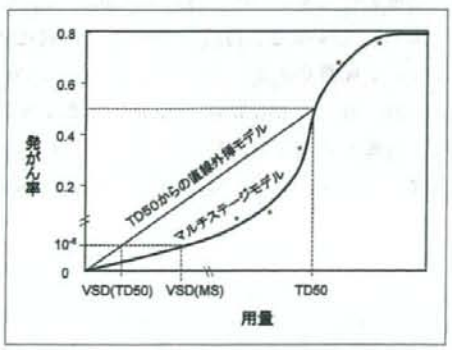

図3 Virtually Safety Doseの算出法

しかしながら，TTC レベルはその発がん物質に遺伝毒性があった場合にはより慎重な取り扱いが必要となる．Kroes らは 600 以上の発がん化学物質を比較して，TTC を 1.5 μg/人とした場合，遺伝毒性もしくは要注意構造を持つ遺伝毒性物質の幾つかについて高い発がんリスクを懸念している．[2] このため，多くの専門家は食事中に低レベルで存在する遺伝毒性／要注意構造を持つ発がん物質に関しては TTC を 1 桁低い 0.15 μg/人とすることを推奨している．さらに，TTC が適応できないような極めて強力な遺伝毒性発がん物質としてアフラトキシン類，アゾキシ化合物，ニトロソ化合物を挙げている．これら化合物に関しては個別の毒性データとリスク管理が必要であり，TTC を適用すべきではない．

一方，医薬品に関しては別の TTC の考え方がある．医薬品そのものに遺伝毒性があることは許されないが，そこに含まれる不純物に遺伝毒性がある場合，TTC の概念を取り入れた不純物のリスク管理が米国，EU でガイドライン化されつつある．これらガイドラインでは場合によっては，不純物に遺伝毒性があっても 1 日あたり 120 μg/人までの TTC が許容される．医薬品は食事と異なり，摂取(服用)期間が限られていること，また医薬品のベネフィットを考慮した 10^{-5} のリスクレベルなどが採用された段階的 TTC が提唱されている．[3]

6 おわりに

発がん率は人口あたりで発生するがん患者の数であり，動物実験による発がん性試験は担がん動物の数によって評価される．その単位は/人口，/動物数であり普遍である．一方，遺伝毒性の単位は試験系によって異なる．エームス試験は/plate，染色体異常試験は/cell，遺伝子突然変異試験は/gene によって評価される．単位が違えばその検出レベルも異なり，そこで仮に閾値が観察されたとしても，その値は試験系に依存する．また，発がん性は種差，個体差等によって変動することは当然考えられるが，遺伝毒性とは「DNA や染色体の構造的もしくは量的変化を引き起こす性質」であり，DNA や染色体がすべての生物で共通であることを考慮すると，それは普遍でなくてはならない．もし，遺伝毒性に閾値が存在するのであれば，試験法によってそれが変動すること自体が矛盾である．したがって，遺伝毒性とはそもそも閾値を論じるような性質のものではないといえるのかも知れない．

遺伝毒性発がん物質に無理に閾値を設定し，ゼロリスクを求めるよりも，低レベルのリスクを，無視しうる(negligible)，もしくは許容できる(acceptable)リスクとして評価し，社会が受け入れることの方が現実的と考える．文明社会で生活する限り，多くの化学物質の摂取は不可避であり，そのベネフィットとリスクのバランスを考えることが重要である．また，我々人間は自然の食物からも多くの化学物質を摂取しており，それらが遺伝毒性発がん物質であることも少なくない．これら化学物質の中には，一般化学物質よりも高い 10^{-4}～10^{-5} というリスクレベルでないと管理できないものもある．Doll と Peto が言うようにがんの最大の原因は我々の日常の食べ物にあり，残留農薬や食品添加物にあるのではない．[4] もちろん，これらリスクはできるだけ回避することは必要であるが，やはりここでもバランスが重要である．このバランス感覚を身につけることが，成熟した社会での安心した生活に繋がるものと考える．

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Possible participation of oxidative stress in causation of cell proliferation and *in vivo* mutagenicity in kidneys of *gpt* delta rats treated with potassium bromate

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ABSTRACT

Clarifying the participation of oxidative stress among possible contributing factors in potassium bromate ($KBrO_3$)-induced carcinogenesis is of importance from the perspective of human health protection. In the present study, utilizing the antioxidative effects of α-tocopherol (α-TP) or sodium ascorbic acid (SAA) to attenuate oxidative stress, alterations in bromodeoxyuridine labeling indices (BrdU-LIs) and reporter gene mutations in kidneys of male and female *gpt* delta rats given $KBrO_3$ were examined. Five male and female *gpt* delta rats in each group were given $KBrO_3$ at a concentration of 500 ppm in the drinking water for 9 weeks, with 1% of α-TP or SAA administered in the diet from 1 week prior to the $KBrO_3$ treatment until the end of the experiment. Increases in 8-hydroxydeoxyguanosine levels in kidney DNA of both sexes of rats given $KBrO_3$ were significantly inhibited by SAA, but not α-TP. While BrdU-LIs in the proximal tubules of female rats were also significantly reduced by SAA, those in the males and *gpt* mutant frequencies in kidney DNA of both sexes were not affected by SAA or α-TP. Immunohistochemical and Western blot analyses for α_{2u}-globulin strongly suggested that induction of cell proliferation observed in the males might primarily result from accumulation of this protein, independent of oxidative stress. The overall data indicated that while oxidative stress well correlates with induction of cell proliferation in females, its role in males and in generation of *in vivo* mutagenicity by $KBrO_3$ in both sexes is limited.

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1. Introduction

During the bread making process, bromate is considered to be converted to bromide (Kurokawa et al., 1990), so that use of potassium bromate ($KBrO_3$) has been permitted as a flour improver for bread making in Japan and the USA in spite of its carcinogenicity (Kurokawa et al., 1986; DeAngelo et al., 1998). However, since ozonation of surface water for disinfection yields $KBrO_3$ as a by-product (Cavanagh et al., 1992), there is still concern regarding the human hazard presented by its renal carcinogenicity. As is clear from the specific use as a food additive, $KBrO_3$ is a potent oxidizing agent. This property is responsible for changes in DNA bases as well as lipid peroxidation (LPO), in the kidneys of treated rats (Chipman et al., 1998; Umemura et al., 1998). Since 8-hydroxydeoxyguanosine (8-OHdG), a form of guanine oxidized at C-8 position, is known to be fairly stable (Kasai and Nishimura, 1991), elevation of this oxidized base following $KBrO_3$ exposure implies involvement of oxidative stress in $KBrO_3$-induced carcinogenesis (Umemura and Kurokawa, 2006; Delker et al., 2006). Simultaneous treatment with antioxidants is known to prevent elevation of 8-OHdG and LPO induced by $KBrO_3$ (Cadenas and Barja, 1999; El-Sokkary, 2000), but it remains unclear how oxidative stress contributes to $KBrO_3$-carcinogenesis.

In two-stage model using *N*-ethyl-*N*-hydroxyethyl-nitrosamine as an initiator, $KBrO_3$ enhances renal tumorigenesis in both male and female rats (Kurokawa et al., 1985; Umemura et al., 1995). Also, short-term exposure to $KBrO_3$ in males was found to significantly elevate bromodeoxyuridine-labeling indices (BrdU-LIs) in proximal convoluted tubules (PCTs) in the same dose-dependent manner as evident in the promotion assay (Umemura et al., 1993). As a possible mode of action, we have proposed involvement of α_{2u}-globulin accumulation in $KBrO_3$-induced cell proliferation in males (Umemura et al., 2004). However, the fact that PCT BrdU-LIs in females exposed to $KBrO_3$ were also increased, albeit at higher doses than in males, implies the existence of other causal factors.

A two-stage model using nitrilotriacetate as a promoter has further shown that $KBrO_3$ possesses initiating activity for renal carcinogenesis in male rats (Umemura et al., 2006). In addition to previous positive results in several mutagenicity tests (Ishidate et al., 1984; Ishidate and Yoshioka, 1980; Hayashi et al., 1988), recent findings using isolated rat kidney cells (Nesslany et al., 2007) and

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human peripheral lymphocytes (Kaya and Topaktas, 2007) point to genotoxic potential. Also, in an *in vivo* mutation assay using reporter gene transgenic rats, $KBrO_3$ proved capable of elevating the transgene mutation frequency (Umemura et al., 2006; Yamaguchi et al., 2008). Although induction of micronuclei in rat peripheral blood reticulocytes by $KBrO_3$ was inhibited by antioxidants (Sai et al., 1992), there have been few reports demonstrating clear relationships between oxidative stress and its genotoxicity.

Assessment of the participation of oxidative stress in $KBrO_3$ carcinogenesis is clearly necessary for accurate estimation of its hazard risk to humans. In the present study, taking advantage of the inhibitory effects of two different types of antioxidants, α-tocopherol (α-TP) and sodium ascorbic acid (SAA), changes in BrdU-LIs and α_{2u}-globulin accumulation in PCT, and transgene mutations in kidney DNA of male and female *gpt* delta rats given $KBrO_3$ were investigated.

2. Materials and methods

2.1. Chemicals

$KBrO_3$, α-TP and SAA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Alkaline phosphatase was obtained from Sigma Chemical (St. Loius, MO, USA) and nuclease P1 was from Yamasa Shoyu (Chiba, Japan). Anti-BrdU monoclonal and anti-α_{2u}-globulin polyclonal antibodies were from DakoCytomation (Glostrup, Denmark) and R&D Systems, Ltd. (Minneapolis, MN, USA), respectively.

2.2. Animals, diet and housing conditions

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five-week-old male and female *gpt* delta F344 rats carrying about five tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan). They were housed in polycarbonate cages (5 rats per cage) with hardwood chips for bedding in a conventional animal facility, maintained under conditions of controlled temperature (23 ± 2 °C), humidity (55 ± 5%), air change (12 times per hour), and lighting (12 h light/dark cycle) and were given free access to CRF-1 basal diet (BD; Charles River Japan) and tap water.

2.3. Animal treatments

Groups of 5 male and female *gpt* delta rats were administered $KBrO_3$ solution at a concentration of 500 ppm in the drinking water for 9 weeks. Additional subgroups of 5 male and female *gpt* delta rats were fed α-TP or SAA at a dose of 1% in the diet from 1 week prior to the $KBrO_3$ treatment until the end of the experiment. Further groups of 5 male and female rats each were given basal diet and distilled water (DW) throughout the experimental period as controls. All animals were injected with BrdU (100 mg/kg) i.p. twice a day for the final 2 days of the exposure and once on the day of termination, 2 h before killing. At the end of each period, the animals were killed under ethyl ether anesthesia and a part of left kidney was homogenized in Isogen (Nippon Gene, Tokyo, Japan) and stored at −80 °C until use for isolation of total RNA. The remaining left kidney was also stored at −80 °C for 8-OHdG measurement, Western blot analysis and *in vivo* mutation assays. Portions of right kidneys were fixed in ice-cold acetone for 3 days and processed for embedding in paraffin, sectioning (4 μm), and immunostaining for BrdU after histochemical demonstration of γ-glutamyltranspeptidase (γ-GT) activity. The remaining kidney tissue was fixed in buffered formalin and then routinely processed for embedding in paraffin, sectioning and immunostaining for α_{2u}-globulin.

2.4. Measurement of nuclear 8-OHdG

To prevent 8-OHdG formation as a byproduct during DNA isolation (Kasai, 2002), kidney DNA was extracted by a slight modification of the method of Nakae et al. (1995). Briefly, nuclear DNA was extracted with a commercially available DNA Extractor WB Kit (Wako Pure Chemical Industries, Ltd.) containing an antioxidant NaI solution to dissolve cellular components. For further prevention of autooxidation in the cell lysis step, deferoxamine mesylate (Sigma Chemical Co.) was added to the lysis buffer (Helbock et al., 1998). The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase, and levels of 8-OHdG (8-OHdG/10^5 deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II, ESA, Bedford, MA, USA).

2.5. Immunohistochemical procedures

For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (1:100), biotin-labeled horse anti-mouse IgG (1:400), and avidin-biotin-peroxidase complex (ABC) after denaturation of DNA with 4N HCl. Before the denaturation step, sections were processed histochemically for demonstration of γ-GT activity by the method of Rutenburg et al. (1969) using L-glutamyl-4-methoxy-β-naphtylamide (Polysciences Ltd., Warrington, PA, USA) as the substrate in order to assist in distinguishing the three kinds of tubules, as previously described (Umemura et al., 1992). The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). For immunohistochemical staining of α_{2u}-globulin, sections were treated sequentially with normal goat serum, polyclonal rabbit anti-α_{2u}-globulin (1:100), biotin-labeled goat anti-rabbit IgG (1:400), and ABC after denaturation of DNA with 4N HCl. The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

2.6. Cell proliferation quantification

At least 3000 tubule cells in each kidney were counted and BrdU-LIs were calculated as the percentages of cells positive for BrdU incorporation.

2.7. Western blotting for α_{2u}-globulin

Kidney samples were homogenized with a Teflon homogenizer in ice-cold 50 mM Tris–HCl, pH 7.4 containing 0.25 M sucrose and a 1% protease inhibitor cocktail (Sigma Chemical Co.). The homogenate was centrifuged for 10 min at 10,000 × *g*, 4 °C, and the resulting supernatant was collected. Protein concentrations were determined with a BCA Protein Assay kit (Pierce Biotechnology Ltd., Rockford, IL, USA). The samples containing 20 μg protein were resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) and analyzed with anti-α_{2u}-globulin (1:200), as well as anti-β-actin as a loading control (1:8000, Sigma Chemical Co.). Appropriate peroxidase-conjugated secondary antibodies (1:2000, Dako Cytomation) were used to detect proteins with ECL Plus (Amersham Bioscience Corp., Piscataway, NJ, USA) reagents.

2.8. In vivo mutation assays

6-TG and Spi$^-$ selections were performed as previously described (Umemura et al., 2007) using the first three animals each group. Briefly, genomic DNA was extracted from the kidneys of the first 3 animals in each group, and lambda EG10 DNA (48 kb) was rescued as phages by *in vitro* packaging.

For 6-TG selection, packaged phages were incubated with *Escherichia coli* YG6020, which expresses Cre recombinase, and converted to plasmids carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, 3000-fold diluted phages were used to infect YG6020, and poured on plates containing chloramphenicol without 6-TG. The plates were then incubated at 37 °C for selection of 6-TG-resistant colonies. Positively selected colonies were counted on day 3 and collected on day 4. The mutant frequency (MF) was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For Spi$^-$ selection, packaged phages were incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar plates. Next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipetters and the agar plugs were suspended in SM buffer. In order to confirm the Spi$^-$ phenotype of candidates, the suspensions were spotted on three types of plates where XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with soft agar. Real Spi$^-$ mutants, which made clear plaques on every plate, were counted.

For characterizing the mutation spectra of *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously (Nohmi et al., 2000). DNA sequencing was performed with the Big Dye™ Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM™ 310 Genetic Analayzer (Applied Biosystems).

3. Results

As shown in Fig. 1, 8-OHdG levels in kidney DNA of male and female *gpt* delta rats given $KBrO_3$ were significantly increased as compared to the controls. Although the levels in *gpt* delta rats of both sexes co-treated with α-TP or SAA were still significantly higher than the controls, significant decreases in either sex of rats were evident as compared to $KBrO_3$-treated animals.

PCT BrdU-LIs in male and female gpt delta rats exposed to $KBrO_3$, with or without antioxidants, are shown in Fig. 2. In the males, $KBrO_3$ exposure induced prominent rise of BrdU-LIs with statistical significance, which was not affected by α-TP or SAA treatment. In the females, $KBrO_3$ significantly increased BrdU-LIs as in the males,

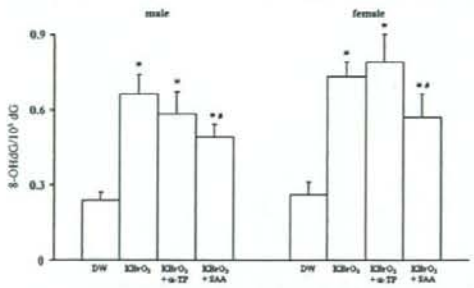

Fig. 1. 8-OHdG levels in kidneys of male and female *gpt* delta rats co-treated with $KBrO_3$ and α-TP or SAA. Values are means ± SDs of data for 5 rats. $^*p < 0.01$, significantly different from the controls (DW). $^\#p < 0.01$, significantly different from the $KBrO_3$ alone group.

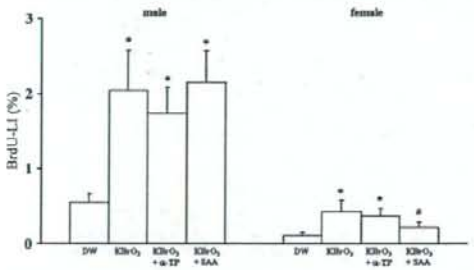

Fig. 2. BrdU-LIs for proximal convoluted tubules (PCT) of male and female *gpt* delta rats co-treated with $KBrO_3$ and α-TP or SAA. Values are means ± SDs of data for 5 rats. $^*p < 0.01$, significantly different from the controls (DW). $^\#p < 0.01$, significantly different from the $KBrO_3$ alone group.

but in this case, co-treatment with SAA, but not α-TP, was associated with suppression of the elevation.

Immunohistochemical data for α_{2u}-globulin are shown in Fig. 3(A–E). Because α_{2u}-globulin is a male rat-specific urinary protein, in controls scattered accumulation was limited to males (Fig. 3 A). $KBrO_3$ caused accumulation of the protein only in males (Fig. 3 B), which was not inhibited by any antioxidant treatments (Fig. 3 C and D), no binding being evident even in the $KBrO_3$-treated female rats (Fig. 3 E). These findings were directly in line with Western blot results for α_{2u}-globulin (Fig. 3 F).

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Tables 1 and 2. In the males, although statistical analysis could not be performed because no *gpt* mutant colonies were detected in one control rat, MFs in all the treated groups showed a tendency for increase (Table 1). Likewise, in the females, elevation of MFs in all the treated groups were found, the increase in the $KBrO_3$ alone group being statistically significant (Table 2). To characterize *gpt* mutations DNA sequencing was performed (Table 3). Among the groups in which the MFs were significantly increased, there were no common types of mutations. GC:AT transitions in α-TP treated males, GC:TA and AT:TA transversions in SAA treated males and single base deletions in $KBrO_3$ alone treated females showed the highest mutation frequencies. As shown in Table 4, there were no changes in Spi^- MFs in males. In all the treated females, a tendency for elevation of Spi^- MFs was apparent, with statistical significance in the α-TP treatment case (Table 5). However, co-treatment with the antioxidants did not appear to exert any effects on MFs for the *gpt* gene in the kidneys of rats given $KBrO_3$.

4. Discussion

In the present study, increases of 8-OHdG levels in kidney DNA of male and female rats following $KBrO_3$ exposure were significantly suppressed by SAA, but not α-TP. Although precise mechanisms responsible for the differences in efficacy between the two antioxidants remain to be determined, it has been reported that dietary ascorbic acid is capable of accumulating more effectively in renal cortical tissue of rats than is the case with dietary α-TP (Craven et al., 1997). In consideration of the fact that $KBrO_3$ is efficiently reduced by GSH at brush borders on the luminal surfaces of PCT cells (Murata et al., 2001), eventually yielding oxidative stress (Ballmaier and Epe, 1995, 2006), it is plausible that an aqueous antioxidant would exert preventive effects. Previous study demonstrated that dietary vitamin E inhibited 8-OHdG levels in kidney DNA induced by $KBrO_3$ at higher dose (Cadenas and Barja, 1999). The incompatible results might involve differences in the nature of damage to DNA produced by low vs. high doses of $KBrO_3$. In the present study, simultaneous treatment with SAA was in fact able to attenuate oxidative damage caused by $KBrO_3$.

$KBrO_3$ at a concentration of 500 ppm has been reported to promote tumor development in the rat kidney of both sexes (Kurokawa et al., 1985; Umemura et al., 1995). Induction of cell proliferation, regarded as a contributing factor, was observed even at 30 ppm of $KBrO_3$ in males, in contrast to the lowest effective dose in females being 250 ppm (Umemura et al., 2004). Interestingly, α_{2u}-globulin accumulation in the kidneys of male rats also occurred in a

Table 1
Effects of antioxidants on *gpt* mutant frequencies in the kidneys of male *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
		1	6.0	0[a]	0	
DW	BD	2	9.3	1	0.11	0.12
		3	7.7	2	0.26	
		6	9.2	3	0.33	
$KBrO_3$	BD	7	98.8	2	0.20	0.43 ± 0.29
		8	6.6	5	0.76	
		11	8.7	8	0.92	
$KBrO_3$	α-TP	12	5.4	8	1.48	1.14 ± 0.30
		13	9.7	10	1.03	
		16	10.1	3	0.30	
$KBrO_3$	SAA	17	7.8	6	0.77	0.53 ± 0.24
		18	9.3	5	0.53	

DW: Distilled water, BD: basal diet.
[a] Two colonies were found on the plate, but neither harbored any *gpt* mutations.

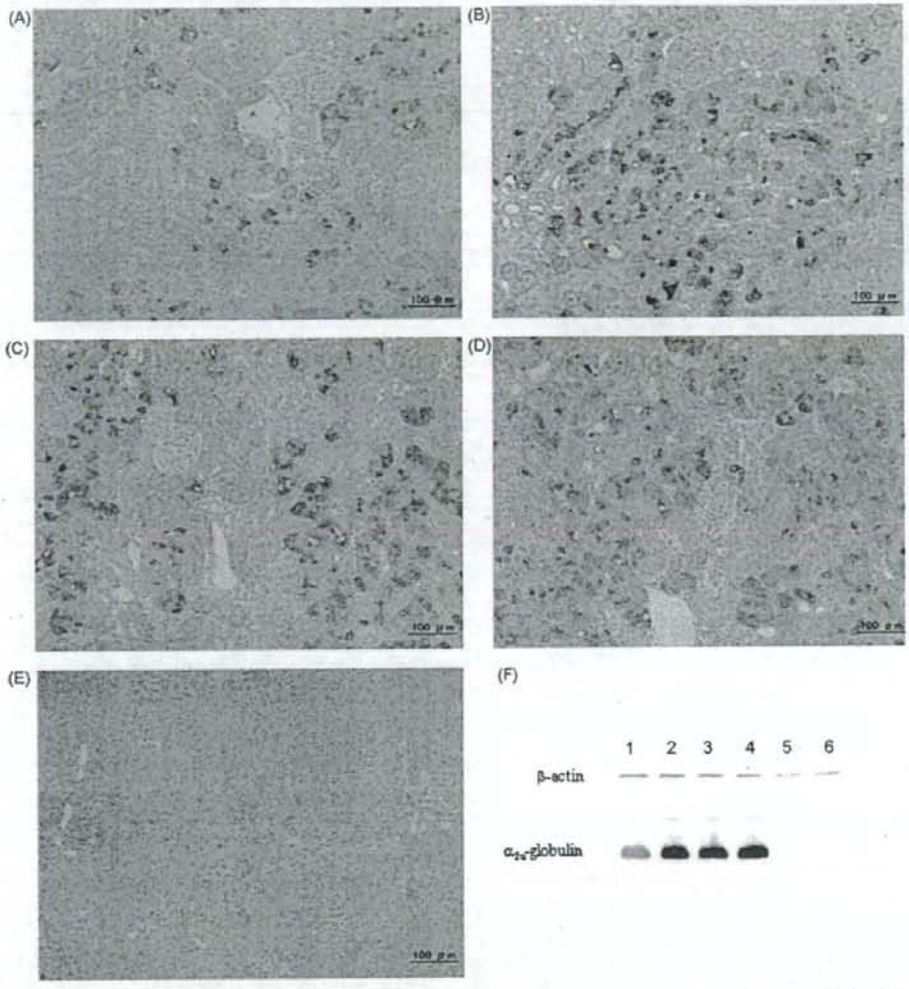

Fig. 3. Photomicrographs of immunohistochemical staining for α_{2u}-globulin in the kidneys of male (A–D) and female (E) *gpt* delta rats given DW (A), $KBrO_3$ (B), $KBrO_3$ and α-TP (C), $KBrO_3$ and SAA (D), or $KBrO_3$ (E). Western blot analysis of α_{2u}-globulin (F) from kidneys of male (lanes 1–4) and female (lanes 5 and 6) *gpt* delta rats given DW (lane 1), $KBrO_3$ (lane 2), $KBrO_3$ and α-TP (lane 3), $KBrO_3$ and SAA (lane 4), DW (lane 5), and $KBrO_3$ (lane 6). α_{2u}-globulin accumulation is more prominent in $KBrO_3$-treated male rats (B) as compared to the controls (A), and is not affected by α-TP (C) or SAA (D) treatment. Note the lack of accumulation in $KBrO_3$-treated females (E), in line with the Western blot analysis (F).

dose-dependent manner from 30 ppm, the protein levels being statistically significant at 125 ppm and above (Umemura et al., 2004). Therefore, it is very likely that this protein accumulation is involved in the cell proliferation observed in the males. In the present study, immunohistochemical and Western blot analysis of α_{2u}-globulin clearly demonstrated accumulation due to $KBrO_3$ exposure, which was not affected by either of antioxidants. This might account for the finding that simultaneous administration of SAA failed to block the rise in BrdU-LIs in males exposed to $KBrO_3$. In general, non-covalently binding of chemicals to the α_{2u}-globulin binding site, a highly aromatic region of the α_{2u}-globulin binding pocket (Huwe et al., 1996) seems to be an initial step, followed by accumulation of the protein in lysosomes of PCT because of resultant resistance to proteolysis. Alternatively, since activities of cystein proteases in lysosomes are prerequisite for degradation of α_{2u}-globulin (Saito et al., 1992), there might be the possibility of primary decrease of protease activity in lysosomes due to interaction of $KBrO_3$ with their thiols acting as a trigger for accumulation (Read, 1991). Although it remains uncertain whether $KBrO_3$ has affinity for the pocket or detrimental effects on lysomal functions, our data imply that induction of cell proliferation following accumulation of the protein in males occurs independently of oxidative stress. On the other hand, in the females lacking α_{2u}-globulin, the BrdU-LI elevation in PCT of rats given $KBrO_3$ was alleviated by SAA. Since mRNA levels of oxidative stress-related genes such as *c-fos*, *c-jun* and *NF-κB* were not elevated in kidneys of $KBrO_3$-treated female rats (data not shown), further studies appear warranted to determine links at the molecular level between oxidation and cell proliferation.

Exposure of female *gpt* delta rats with a genetic background of F344 to $KBrO_3$ at 500 ppm for 9 weeks induced significant elevation of *gpt* MFs along with Spi^- MFs. However, the antioxidants were unable to prevent any type of mutation. 8-OHdG is not only a

Table 2
Effects of antioxidants on *gpt* mutant frequencies in the kidneys of female *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
DW	BD	51	9.9	3	0.30	0.24 ± 0.07
		52	6.0	1	0.17	
		53	11.8	3	0.25	
$KBrO_3$	BD	56	11.0	7	0.64	0.53 ± 0.11*
		57	9.6	4	0.42	
		58	11.4	6	0.53	
$KBrO_3$	α-TP	61	8.6	6	0.70	0.48 ± 0.20
		62	9.5	4	0.42	
		63	13.0	4	0.31	
$KBrO_3$	SAA	66	6.8	2	0.29	0.44 ± 0.34
		67	10.2	2	0.20	
		68	8.4	7	0.83	

DW: Distilled water, BD: basal diet.
* $p < 0.01$ vs. DW/BD.

Table 3
Mutation spectra of *gpt* mutant colonies.

Sex	Male				Female			
Treatment (water/diet)	DW/BD	$KBrO_3$/BD	$KBrO_3$/α-TP	$KBrO_3$/SAA	DW/BD	$KBrO_3$/BD	$KBrO_3$/α-TP	$KBrO_3$/SAA
Base substitution								
Transversions								
GC:TA	0[a]	1(0.04)	4(0.17)	3(0.11)	2(0.07)	1(0.03)	0	2(0.08)
GC:CG	0	0	0	1(0.04)	0	1(0.03)	1(0.03)	1(0.04)
AT:TA	0	2(0.08)	4(0.17)	3(0.11)	0	3(0.09)	4(0.13)	2(0.08)
AT:CG	0	1(0.04)	2(0.08)	0	0	3(0.09)	0	0
Transitions								
GC:AT	2(0.09)	1(0.04)	8(0.34)	2(0.07)	2(0.07)	4(0.13)	6(0.19)	3(0.12)
AT:GC	0	1(0.04)	3(0.13)	1(0.04)	0	1(0.03)	1(0.03)	0
Deletion								
Single bp	0	1(0.04)	3(0.13)	1(0.04)	2(0.07)	6(0.19)	2(0.06)	1(0.04)
Over 2 bp	0	1(0.04)	2(0.08)	2(0.07)	1(0.04)	1(0.03)	0	1(0.04)
Insertion	0	1(0.04)	0	0	0	0	1(0.03)	1(0.04)
Complex	1(0.04)	1(0.04)	0	0	0	0	1(0.03)	0
Total	3(0.13)	10(0.39)	26(1.09)	14(0.51)	7(0.25)	20(0.63)*	14(0.51)	11(0.63)

DW: Distilled water, BD: basal diet. Values appearing in parenthesis indicates mutation frequency, $\times 10^{-5}$. *$p < 0.01$ vs. DW/BD.
[a] The number of colonies with independent mutations.

representative marker for oxidative stress but also a primary cause of GC:TA transversions due to mispairing with A (Cheng et al., 1992; Shibutani et al., 1991). Nevertheless, spectrum analysis of the *gpt* mutants induced by $KBrO_3$ did not indicate a majority of GC:TA transversions. Instead, deletions were most common in concord with the results of Spi^- mutation assays. In our previous study using male *gpt* delta rats with a Sprague–Dawley genetic background, significant elevation of Spi^- MFs was rather apparent (Umemura et al., 2006). Furthermore, in an *in vitro* genotoxicity assay for $KBrO_3$ using human lymphoblastoid TK6 cells (Luan et al., 2007) or mouse lymphoma cells (Harrington-Brock et al., 2003), $KBrO_3$ induced large deletions, including loss of heterozygosity at

Table 4
Effects of antioxidants on *red/gam* mutant frequencies in the kidneys of male *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2) (Spi^-)	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
DW	BD	1	19.2	8	0.42	0.82 ± 0.52
		2	20.3	13	0.64	
		3	17.8	25	1.40	
$KBrO_3$	BD	6	15.3	36	2.36	1.04 ± 1.15
		7	21.8	8	0.37	
		8	15.7	6	0.38	
$KBrO_3$	α-TP	11	23.4	9	0.39	0.43 ± 0.05
		12	26.0	11	0.42	
		13	14.3	7	0.49	
$KBrO_3$	SAA	16	17.8	9	0.51	0.57 ± 0.08
		17	14.5	8	0.55	
		18	19.5	13	0.67	

DW: Distilled water, BD: basal diet.

Table 5
Effects of antioxidants on *red/gam* mutant frequencies in the kidneys of female *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2) (Spi$^-$)	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
		51	15.2	2	0.13	
DW	BD	52	6.8	5	0.73	0.45 ± 0.30
		53	10.5	5	0.48	
		56	5.2	6	1.16	
$KBOrO_3$	BD	57	4.9	2	0.41	0.89 ± 0.42
		58	6.4	7	1.09	
		61	5.6	7	1.24	
$KBrO_3$	α-TP	62	8.4	8	0.96	1.02 ± 0.20*
		63	3.5	3	0.85	
		66	5.2	8	1.55	
$KBrO_3$	SAA	67	7.0	5	0.71	0.97 ± 0.51
		68	7.8	5	0.64	

DW: Distilled water, BD: basal diet.
* $p < 0.05$ vs. DW/BD.

TK locus, but not GC:TA transversions. On the other hand, in the present study, Spi$^-$ MFs in males were not increased, in contrast to the previous study demonstrating significant increment. Although certain differences between genetic backgrounds should not be ignored, seemingly inconsistent results might reflect smaller increase of MFs following $KBrO_3$ exposure (2–3 fold) as compared to the case (10–30 fold) with potent genotoxic carcinogens (Kanki et al., 2005). In other words, as shown in microbial and the *Hprt* mutation assays in mammalian cells (Speit et al., 1999), the potential of $KBrO_3$ to induce mutations may be very weak (Harrington-Brock et al., 2003). Actually, we obtained negative data for transgene mutations at 250 ppm for 13 weeks (Umemura et al., 2006) and another group similarly reported negative findings with 125 ppm for 16 weeks (Yamaguchi et al., 2008). The hypothesis of weak mutagenicity is strongly supported by a bioassay showing $KBrO_3$ at 500 ppm for 13 weeks to be incapable of effecting tumor development with appropriate promotion, despite preneoplastic lesions being enhanced (Umemura et al., 2006).

In conclusion, oxidative stress generated by $KBrO_3$ might take part in induction of cell proliferation in PCT of female rats, leading to tumor promoting potential. In males, in contrast, α_{2u}-globulin accumulation independent of oxidative stress plays a major role in cell proliferation, which implies that the tumor promotion observed in males is not directly comparable to the human situation. Likewise, induction of reporter gene mutations by $KBrO_3$ is unlikely to be due to oxidative stress, the extent of which being much lower as compared to that of potent genotoxic carcinogens. The data overall allow us to speculate that the predominant contributing factor for $KBrO_3$-induced renal carcinogenesis is tumor promoting potential, which is only to a limited extent associated with oxidative stress.

Conflict of interest

None.

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—Minireview—

Spontaneous Mutagenesis in Rodents: Spontaneous Gene Mutations Identified by Neutral Reporter Genes in *gpt* Delta Transgenic Mice and Rats

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Transgenic rodents are valuable models for investigation of genotoxicity of chemicals *in vivo*. We have developed *gpt* delta transgenic mice (C57BL/6J background) and rats (Sprague-Dawley, SD), which have the ability to identify both point mutations by the *gpt* assay [6-thioguanine (6-TG) selection] and certain types of deletions by the Spi$^-$ (Spi, sensitive to P2 interference) assay. Recently, the *gpt* delta SD rat was backcrossed with the Fisher 344 (F344) rat to establish an *gpt* delta F344 rat. The average spontaneous *gpt* mutation frequencies (MFs) are about 4.5×10^{-6} in both SD and F344 *gpt* delta rats as well as in *gpt* delta mice. The G:C to A:T transitions at 5′-CpG-3′sites and G:C to T:A transversions are the predominant spontaneous *gpt* mutations in rats and mice. However, there is one false mutation (*e.g.* A:T to T:A at position 299) in the rats. The base substitution may have arisen when the lambda EG10 transgene was introduced into the genome of the SD rat during transgenesis. In the Spi$^-$ assay, 1-bp deletions in repetitive sequences are predominantly observed in both mice and rats. Possible mechanisms underlying the spontaneous mutations in *gpt* delta rodents are discussed.

Key words—*gpt* delta transgenic rodent, spontaneous mutation, mutation spectrum, *gpt* assay, Spi$^-$ assay

OVERVIEW OF *gpt* DELTA TRANSGENIC RODENTS

Gene mutations play an important role in the etiology of many human diseases including cancer. Since humans are exposed to a variety of endogenous and exogenous mutagens, there has been considerable interest in the relationship between exposure, genotoxic effects, and cancer incidence. To assess the risk of mutagens to the human genome, genotoxicity tests have been developed, including *in vivo* mutation assays using experimental animals, which play a crucial role in risk assessment. To investigate *in vivo* genotoxicity, a number of transgenic rodent mutation assays have been developed by introducing reporter transgenes into the chromosome of every cell of the animal.[1,2] Using these systems, mutagenic events induced in a rodent can be detected by recovering the transgene and analyzing the phenotype of the reporter gene in a bacterial host. These models permit quantitation of mutations and identification at the sequence level in any tissue or organ in the body. *lacZ*, *lacI* or *cII* have been employed as reporter genes in transgenic rodents, such as the Muta^{TM} mouse, and the Big Blue^R mouse and rat.[3–7] Despite differences in size and sequence context, spontaneous mutation frequencies of these reporter genes are in the mid-10^{-5} range and those are predominantly base substitutions in most tissues. This high background of base substitutions may make it difficult to detect rare mutations such as deletions induced by ionizing radiation.[8,9] To overcome this limitation, a transgenic "*gpt* delta" assay system has been developed for the efficient detection of both point mutations and deletions.[1,10] A unique feature of the lambda EG10 phage vector constructed for this system is the incorporation of two different positive selection methods: the *gpt* assay [6-thioguanine (6-TG) selection] using the *gpt* gene of *Escherichia coli* (*E. coli*) that detects mainly point mutations such as base substitutions and frameshifts, and the Spi$^-$ (Spi, sensitive

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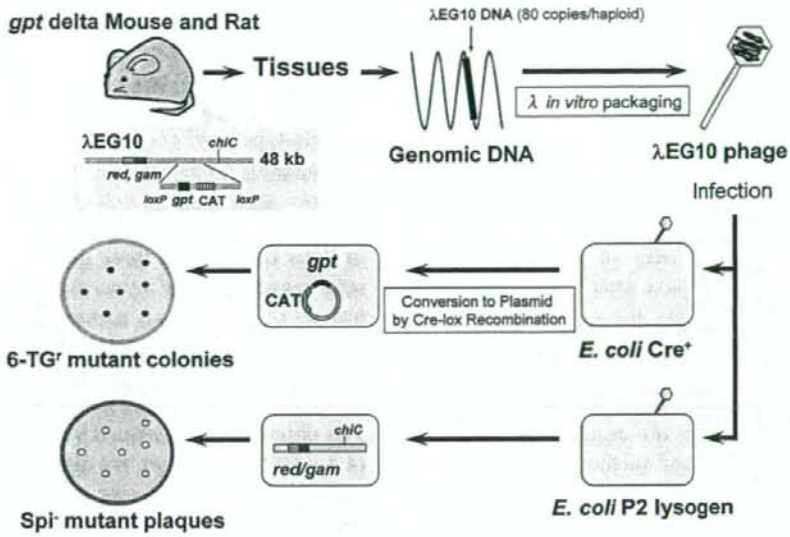

Fig. 1. Principle of *gpt* Delta Transgenic Rodent Mutation Assays[1, 10, 13, 14]

Genomic DNA is extracted from tissue of a transgenic rodent. Lambda EG10 DNA is recovered by *in vitro* packaging and rescued as phage particles. Two distinct *E. coli* host strains are infected with the rescued lambda EG10 phages: one is *E. coli* YG6020 expressing Cre recombinase for the *gpt* assay and the other is a P2 lysogen for the Spi⁻ assay. In the *gpt* assay, lambda EG10 DNA is converted to plasmid carrying *gpt* and CAT by cre-lox recombination. Bacteria harboring the plasmids carrying mutant *gpt* are positively selected as colonies on plates containing chloramphenicol and 6-thioguanine. In the Spi⁻ assay, mutant lambda EG10 phages lacking *red/gam* gene functions are positively selected as Spi⁻ plaques on lawns of P2 lysogens. The same DNA prepared from identical tissue sample is applied to both *gpt* and Spi⁻ assays.

to P2 interference) assay (Spi⁻ selection) using the *red/gam* genes of lambda phage that detects deletions including frameshifts (Fig. 1).

To accomplish the *gpt* and Spi⁻ assay, the *gpt* delta transgenic mouse has been developed.[1, 10] This mouse was established by microinjection of lambda EG10 phage DNA into the fertilized eggs of C57BL/6J mice. It carries about 80 copies of the transgene in a head to tail fashion at a single site in chromosome 17 and is maintained as a homozygote (carrying 160 copies of transgene per diploid).[11] The same lambda EG10 transgene used in the *gpt* delta mouse was integrated into the genome of Sprague-Dawley (SD) rat to establish the *gpt* delta rat.[12] The *gpt* delta rat harbors about 5–10 copies of the transgene in chromosome 4 and is maintained as a heterozygote. The *gpt* and Spi⁻ assay systems have been validated primarily in mice with many chemical mutagens/carcinogens, UV and ionizing radiation, for which mutagenicity, organ specificity and mutation spectrum have been thoroughly characterized.[1, 10–28] Recently, the outbred *gpt* delta SD rat was backcrossed with Fisher 344 (F344) rat, to establish an inbred *gpt* delta rat (F344). In this review, we focus on the spontaneous mutations detected by the *gpt* and Spi⁻ assays in *gpt* delta mice and rats and discuss possible mechanisms underlying these *in vivo* mutations.

gpt ASSAY (6-TG SELECTION) FOR POINT MUTATIONS

The principles and method of the *gpt* assay have been described previously (Fig. 1).[1, 10, 15] Briefly, the *gpt* gene encodes guanine phosphoribosyltransferase that is involved in the purine salvage pathway of *E. coli*. This enzyme phosphoribosylates not only guanine, but also 6-TG, which is toxic to cells when it is incorporated into DNA. Thus, *E. coli* cells expressing wild-type *gpt* cannot survive on the plates containing 6-TG. Only *gpt* mutants can form colonies on plates containing 6-TG. The coding region of *gpt* is 456 bp, which is convenient for sequence analysis of the mutants. When *E. coli* strain YG6020 expressing Cre recombinase is infected with lambda EG10 rescued from *gpt* delta rodents, the plasmid region is efficiently excised, circularized and propagated as multi-copy number plasmid carrying *gpt* and chloramphenicol acetyltransferase

(CAT). Bacteria harboring mutated *gpt* genes can be positively selected as colonies on plates containing 6-TG and chloramphenicol (Cm). The number of rescued phages can be determined by plating the cells on the plates containing Cm alone. The *gpt* mutant frequency (MF) is calculated by dividing the number of the *gpt* mutant colonies by the number of rescued Cm-resistant colonies.

The spontaneous *gpt* MFs of *gpt* delta mice (C57BL/6J background) have been previously reported. In most experiments, the values of spontaneous *gpt* MFs are in the range of 5×10^{-6}. This is less than that of other transgenic rodent mutation assay systems, such as Muta mice and Big Blue mice, in which spontaneous MFs are usually greater than 1×10^{-5}. Different selection method and sequence context of the *gpt* gene might account for the lower MF of the *gpt* assay. Even if some types of mutation occurred in the *gpt* gene, residual enzymatic activity may still catalyze enough amount 6-TG to kill the cells. Because the *gpt* mutant cells are grown on M9 minimal medium agar plate containing 6-TG for 3–4 days, phenotypic selection of 6-TG resistance could require complete loss of enzymatic activity of the mutated *gpt* gene product as a selectable phenotype. There are no clearly observed tissue differences in the spontaneous MFs in *gpt* delta mice. Although the number of studies in which multiple tissues types have been analyzed is limited, similar spontaneous MFs were observed in six tissues (liver, spleen, colon, testis, brain and bone marrow) collected from the same animals.[11] No significant differences between male and female were observed in the spontaneous and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced MFs in liver and colon.[11] In *gpt* delta mice, spontaneous MF in the liver at 85 weeks of age increased by a factor of 2 over that at 19 weeks of age.[22] It is also reported that spontaneous MFs increase with age in most somatic cells in Muta mice and Big Blue mice.[29–32] However, the trend could be different in brain and germ tissue, where MFs don't increase with age in adult mice.[33–35] Interestingly, it has been reported that some gene knockout mice show different spontaneous MFs. Spontaneous *gpt* MF in the liver is significantly higher in *ogg1* gene knockout mice, which lack 8-oxo-guanine DNA glycosylase activity.[36] Interleukin-10 (IL-10)-deficient mice, which spontaneously develop intestinal inflammation, have a *gpt* mutation frequency in the colon about five times higher than that in wild type mice.[37] In transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2)-null mice, the spontaneous *gpt* mutation frequency in the lung was approximately three times higher in *nrf2*-null (*nrf2*−/−) mice than in *nrf2* heterozygous (*nrf2*+/−) or wild-type (*nrf2*+/+) mice; whereas in the liver, the mutation frequency was higher in *nrf2*−/− and *nrf2*+/− mice than in *nrf2*+/+ mice. In contrast, no difference in mutation frequency was observed in testis between the three genotypes.[38] These results suggest that the intracellular environment contributes to spontaneous mutations, perhaps through oxidative stress and/or detoxification systems.

In the *gpt* delta rat, the spontaneous *gpt* MFs we have obtained from more than 40 samples from various organs were between 0.9×10^{-6} and 8.5×10^{-6} (4.5×10^{-6} in average). No difference was observed between outbred SD rats (4.5×10^{-6}, calculated from 31 samples) and inbred F344 rats (4.4×10^{-6}, calculated from 10 samples). Although the data represent males and females, different organs (liver, kidney and mammary gland), and different ages (between 10 and 52 weeks old), these values are comparable to those in mice. On the other hand, Hayashi *et al.* reported that the spontaneous *gpt* MF in the liver of the rats was lower than that of mice.[12] The similar observation was also reported in Big Blue mice and rats.[7] Additional studies are needed to investigate the effect of genetic strain background, tissue type, and age in *gpt* delta rat in order to validate these findings.

Spontaneous *gpt* mutation spectra of *gpt* delta rodents are shown in Table 1. Regardless of species or strain background, the proportions of mutations are remarkably similar, although the sample size for F344 rats is small. The most frequent mutations are G:C to A:T transitions. More than half of these occur at 5′-CpG-3′ sequences in mice and SD rats. This suggests that deamination of methylated cytosines at CpG sites contributes to spontaneous mutations in the *gpt* gene *in vivo*.[39] Beside transitions, G:C to T:A transversions are frequently observed base substitutions. This type of mutations may reflect oxidative damage in DNA, such as 8-oxoguanine lesions[40] or abasic sites.[41] Other mutations were frameshifts, short deletions and insertions. These findings were largely consistent between different tissues. Although lower spontaneous *gpt* MFs than Muta mouse and Big Blue mouse are observed, the characteristic mutation spectra are similar, and the similarity of spontaneous mutation spectra in different tissues and different rodent strains are also observed with the