mediated by a feedback signaling pathway involving protein kinase C (PKC), p38 mitogen activated protein kinase (p38MAPK), and phospholipase C (PLC).[11,13,25]

One of the possible targets for radioadaptation is oxidative base damage. A low-dose rate whole body γ-irradiation of mice (1.2 mGy/h, total 0.5 Gy) demonstrated the activation of antioxidative enzymes such as MnSOD and catalase in spleen cells, leading to less DNA damage as determined by a comet assay.[26] Furthermore, down-regulation of the human *CDC16* gene that occurs after oxidative stress causes more rapid and efficient repair in adapted (2 cGy pre-irradiated) human lymphoblastoid cells challenged with 4 Gy irradiation.[12] However, oxidative base excision repair enzymes, including DNA glycosylases, hOGG1, and hNth1, are reportedly not up-regulated at the post-transcriptional level in γ-ray-primed TK6 cells.[27] Those reports suggest that the antioxidant defense machinery is likely to be involved in radioadaptation although the mechanisms involved are still not well understood.

Gene expression also seems to be tightly related to a variety of functions in the adaptive response such as the induction of antioxidant defense machinery, repair of DNA damage, control of cell-cycle progression, *etc.* In fact, *de novo* synthesis of transcripts and proteins is reported to be required for the expression of the adaptive response.[28] Following that report, gene expression analysis has been extensively studied by many investigators.[15,28–31] For example, the *CHD6* gene in human lymphoblastoid cell AHH-1 can be up-regulated by 0.5 Gy of γ-irradiation and its induced expression could be involved in a low-dose hypersensitive response.[29] Recently, gene profiles in the kidney and testis from γ-irradiated (485 days at dose rates of 0.032–13 μGy/min) mice were determined using oligonucleotide microarrays, and differentially expressed genes were identified.[31]

DNA double strand breaks (DSBs) are a most serious type of DNA damage. They can be caused by IR or radiomimetic chemicals, and they can occur spontaneously during DNA replication. The nonrepair or misrepair of DSBs can cause cell death or mutagenic and/or carcinogenic consequences, so the accurate repair of DSBs is important for maintaining genomic integrity.[32,33] In other words, DSB repair is an essential function in all living organisms. Recently analyses using nondividing lymphocyte and fibroblast cells suggested that the adaptive response is not mediated by an enhanced rejoining of DNA strand breaks but rather is a reflection of perturbation in cell cycle progression.[34] On the other hand, the induction of an efficient chromosome repair system by the priming radiation dose is considered to be involved in radioadaptation mechanisms, and in fact, the efficiency of DSB repair in Chinese hamster V79 cells exposed to γ-rays is enhanced by a priming exposure of 5 cGy of γ-rays.[35] The reduced frequencies of chromosomal alterations as described above supports the latter possibility of DSB-repair enhancement. At the present stage, it is difficult to conclude which factor, cell-cycle perturbation or DSB repair, largely contributes to radioadaptation.

THE I-SCEI SYSTEM FOR DSB REPAIR EVALUATION

Outline of the system

A model system was constructed for evaluating DSB repair by tracing the fate of a single DSB on chromosomal DNA. The DSB generated in this system can be considered as a target DNA-lesion susceptible to repair, and this system can distinguish two major DSB repair pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR) (Fig. 1).[36,37] The human lymphoblastoid cell line TSCE5 is heterozygous (+/–) for the thymidine kinase (*TK*) gene and the line TSCER2 is compound heterozygous (–/–; two different TK^- alleles); both carry an I-SceI endonuclease recognition site in intron 4 on one allele of the *TK* gene. DSBs can be generated at the I-SceI site by expression of the I-SceI vector.[36,37] When DSBs occur at the *TK* locus, NHEJ in TSCE5 cells produces TK-deficient mutants while HR between the *TK* alleles in TSCER2 cells produces TK-proficient revertants. This means that positive-negative drug selection for TK phenotypes permits distinction between NHEJ and HR repair.

Cell line construction for use in the system

Details of the strain construction are described in our previous work (Fig. 2).[36] Briefly, in lymphoblastoid TK6 cells heterozygous for the *TK* gene, the functional allele was first inactivated by gene targeting with vector pTK4 to

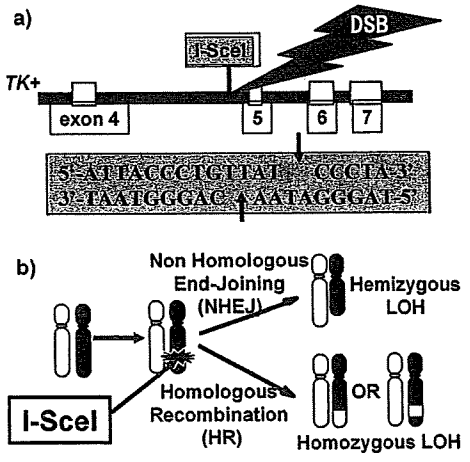

Fig. 1. Principle of DSB formation and repair evaluation. A single DNA double strand break (DSB) is generated at the I-SceI recognition site in a human lymphoblastoid TK6 cell by transfecting an I-SceI expression vector (a) and the efficiencies of DSB repair through non-homologous end-joining (NHEJ) or homologous repair (HR) are evaluated from induction of hemizygous and homozygous LOH events, respectively (see text).

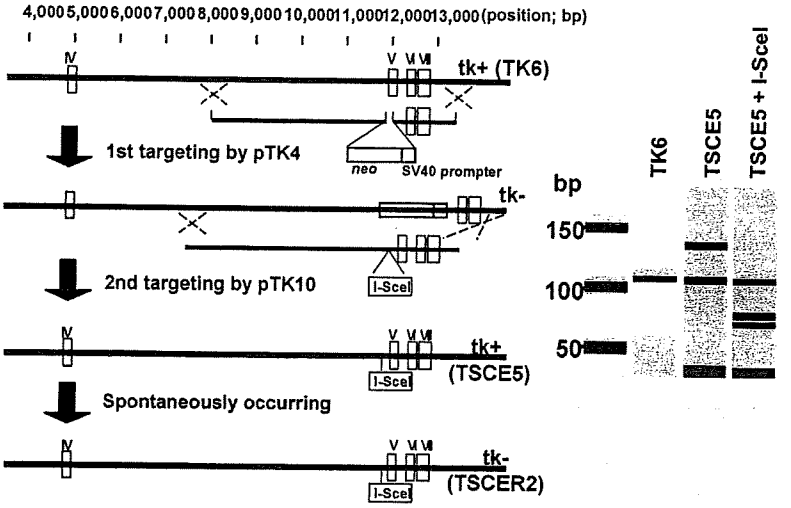

Fig. 2. Cell line construction. In the TK6 cell line, the functional allele of *TK* gene was first inactivated by gene targeting vector pTK4 and then the I-SceI recognition site was introduced at 75 bp upstream of exon 5 in the *TK* gene. The new line was termed TSCE5 and its compound heterozygote (*TK*–/–) cell line, TSCER2, was also isolated (see text).

replace exon 5 of the *TK* gene by a *neo* gene. To introduce the I-SceI recognition site at 75 bp upstream of exon 5, the targeting vector pTK10, encompassing about 6 kb of the original *TK* gene with exons 5, 6, and 7, and the I-SceI recognition site in intron 4, was used to revert the *TK* gene disrupted by pTK4. The new line was termed TSCE5. A spontaneous mutation in a TSCE5 cell (G to A in position 23 of exon 5), which we cloned, led to the compound heterozygote (*TK*–/–) cell line, TSCER2.

I-SceI expression for introduction of DSB

We introduced the I-SceI expression vector (pCBASce) by electroporation methodology using Nucleofector Kit V (amaxa AG, Cologne, Germany) (Fig. 3).[36–38] The I-SceI expression vector was introduced into about 65% of the cells at 24 hr after the transfection and the expression last for 3 days incubation.[37] The relatively long expression allowed us to succeed in estimating the influence of low-dose, low-dose-rate γ-rays irradiation on DSB repair, especially the effect of post-IR-exposure, as described below.

Evaluation of DSB repair efficiencies

Measurements of TK^- mutants and TK^+ revertants allow us to evaluate DSB repair efficiencies through NHEJ and HR pathways, respectively (Fig. 3). In TSCE5, when a DSB at the I-SceI site is repaired by NHEJ involving a deletion in the adjacent exon, the cell can be isolated as a TK-deficient mutant. In TSCER2, when a DSB is repaired by HR between the *TK* alleles, a TK^+ allele can be generated, resulting in a revertant phenotype. The DSB repair *via* NHEJ was 73–86 times higher than that *via* HR in our previous studies.[36,37] These findings are consistent with the report that NHEJ is the major repair pathway in mammalian cells.[39]

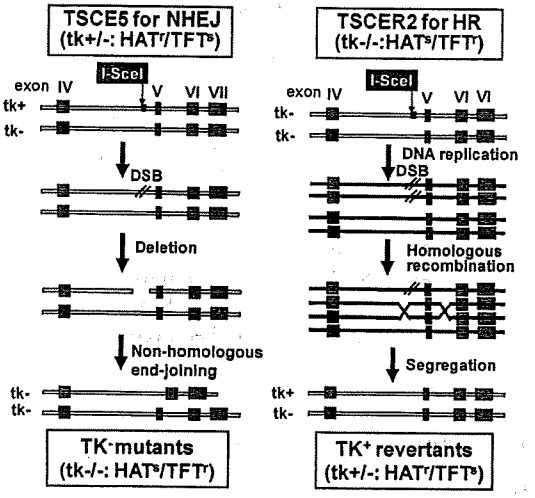

Fig. 3. An approach to evaluate DSB repair efficiency. In TSCE5, when a DSB at the I-SceI site is repaired by NHEJ involving a deletion in the adjacent exon, the cell can be isolated as a TK-deficient mutant. In TSCER2, when a DSB is repaired by HR between the *TK* alleles, a TK^+ allele can be generated, resulting in a revertant phenotype (see text). Filled exons represent TK mutations.

APPLICATION OF THE I-SCEI SYSTEM FOR EVALUATING RADIOADAPTATION IN TERMS OF DSB REPAIR

Influence of low-dose, low-dose-rate γ-rays on DSB repair

The I-SceI digestion system was applied for estimating the influence of low-dose, low-dose-rate γ-irradiation on repair of a site-specifically introduced DSB (Fig. 4).[38] The results

obtained with Mode A (30 mGy of pre-γ-irradiation) and Mode B (8.5 mGy of post-γ-irradiation) are shown in Tables 1 and 2, respectively. The NHEJ repair of DSB was little influenced by either modes of low-dose, low dose-rate γ-irradiation. DSB repair by HR, in contrast, was enhanced by ~50% and ~80% in Mode A and Mode B, respectively. This might implicate that both pre-γ-irradiation (Mode A) and post-γ-irradiation (Mode B) induce a radioadaptation, although both modes of irradiations, especially Mode B, are different from the original concept of radioadaptation. In fact, DSBs are generated during the γ-irradiation in Mode B, because I-SceI expression lasts for 3 days incubation as previously mentioned.

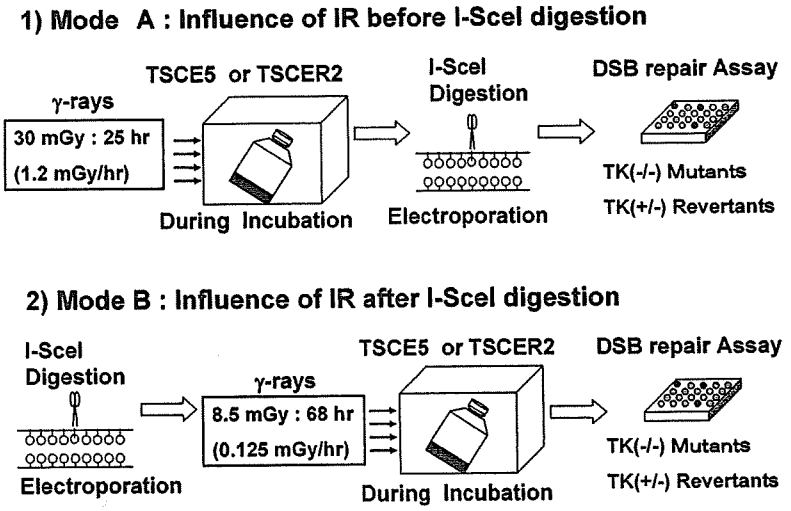

Fig. 4. Influence of low-dose IR exposure on DSB repair. Experimental schemes of radiation exposure and I-SceI expression are illustrated. Mode A: cells were exposed to low-dose, low-dose-rate γ-rays and then transfected with the I-SceI vector by electroporation (see text). 2) Mode B: cells were transfected with the I-SceI vector and then exposed to γ-rays at a much lower dose and dose-rate (see text).

Table 1. Effect of pre-IR exposure on DSB repair (Mode A).

a) NHEJ efficiency in TSCE5 cells

Exp.	Mutant Frequency, MF ($\times 10^{-6}$)				Effect of IR (Relative MF*)
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	3.5	6.1	8600	8500	0.99
2	1.8	3.2	2900	3200	1.1
Average	2.7	4.7	5800	5900	1.0 (P = 0.82)

*Relative MF was calculated as MF (γ-rays + I-SceI)/MF (I-SceI).

b) HR efficiency in TSCER2 cells

Exp.	Revertant Frequency, RF ($\times 10^{-6}$)				Effect of IR (Relative RF*)
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	–	–	90	114	1.3
2	–	–	62	96	1.5
3	–	–	25	45	1.8
Average	–	–	59	85	1.5 (P = 0.021)

*Relative RF was calculated as RF (γ-rays + I-SceI)/RF (I-SceI).

Table 2. Effect of post-IR exposure on DSB repair (Mode B).

a) NHEJ efficiency in TSCE5 cells

Exp.	Mutant Frequency, MF ($\times 10^{-6}$)				Effect of IR (Relative MF*)
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	2.8	1.3	3400	4500	1.3
2	3.1	2.8	12000	17000	1.4
3	–	–	11000	11000	1.0
Average	3.0	2.1	8800	10800	1.2 (P = 0.12)

*Relative MF was calculated as MF (γ-rays + I-SceI)/MF (I-SceI).

b) HR efficiency in TSCER2 cells

Exp.	Revertant Frequency, RF ($\times 10^{-6}$)				Effect of IR (Relative RF*)
	Control	γ-rays	I-SceI	γ-rays + I-SceI	
1	–	–	82	160	2.0
2	–	–	160	270	1.7
3	–	–	110	190	1.7
Average	–	–	120	210	1.8 (P = 0.0013)

*Relative RF was calculated as RF (γ-rays + I-SceI)/RF (I-SceI).

Influence of low-dose X-ray irradiation on DSB repair

We have extensively studied the effects of low-dose IR by using a loss of heterozygosity (LOH) analysis system.[40–42] The thymidine kinase deficient (TK^-) mutants induced in TK6 cells can be classified as LOH type and non-LOH type by this system. The LOH mutants were further classified as homozygous-type and hemizygous-type, and the replaced or deleted part of the chromosome was identified by so-called chromosome mapping. In addition to this kind of analysis at the chromosome level, non-LOH mutants were further characterized at the DNA sequence level to confirm that the mutation occurs in the *TK* gene or not. Recently we could establish the optimum condition for mutagenic radioadaptation in TK6 cells.[43] Under such condition as shown in Fig. 5, the greatest reduction in *TK* mutation frequency was observed in TK6 cells exposed to a challenging X-ray irradiation (2 Gy), and the TK^- mutants so obtained were analyzed by the LOH system.[43]

The TK^- mutation frequency (MF) obtained after the challenging X-ray (2 Gy) exposure, 18.3×10^{-6} was reduced to 11.4×10^{-6} (62% of the original level) by inducing the radioadaptation (50 mGy of pre-X-irradiation at 6 hr before the above challenging X-irradiation; Fig. 6). LOH analysis could classify the TK^- mutational events as non-LOH (mostly mutations in the *TK* gene), hemizygous LOH (deletion of chromosome) and homozygous LOH (homologous recombination [HR] between chromosomes), as mentioned above.[40–42] Non-LOH events are, in theory, classified as chromosomal alterations, but most of non-LOH mutants obtained in this experiment were confirmed to be small mutations in the *TK* gene by DNA base sequencing of mRNA obtained from the mutants.[43] The pre-irradiation decreased the frequencies of non-LOH events and homozygous LOH events to 27% and 60% of the original levels, respectively. The frequency of hemizygous LOH events, however, was not significantly altered by the pre-irradiation. Since LOH events are most likely the consequence of DSB repair, we tried to investigate the influence of priming X-ray irradiation on DSB repair efficiency under the optimum condition for radioadaptation.

The repair efficiency of DSB *via* NHEJ was hardly influenced by the pre-irradiation of 50 mGy X-rays (Table 3). On the other hand, a ~70% enhancement in HR repair of DSB was observed after this treatment. The enhanced activity of HR observed in this experiment could reflect the activity of error-free DSB repair, providing a reduction in genetic alterations at the chromosome level. In fact, we observed a ~60% reduction in the induction of homozygous LOH as mentioned above. The chromosome-mapping analysis demon-

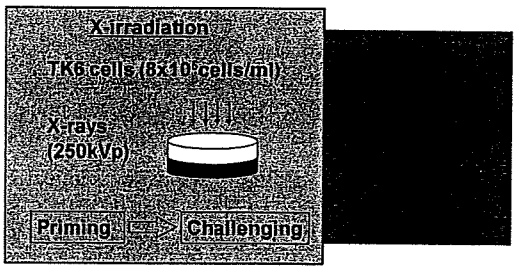

Fig. 5. An experimental scheme for mutagenic radioadaptation. The optimum conditions providing the greatest reduction in the frequency of *TK* mutations induced after a challenging X-ray (2 Gy) irradiation of TK6 cells, are shown in the right panel of this figure. The details have already been described in our previous work.[43]

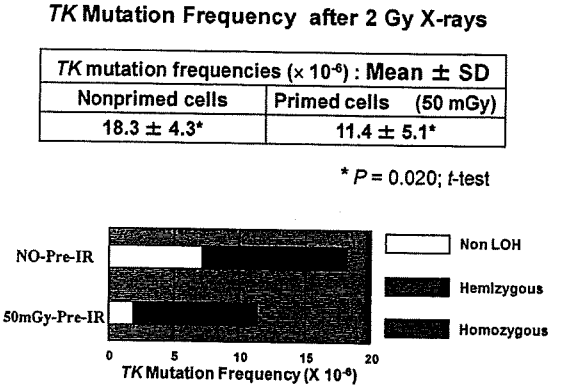

Fig. 6. Genetic analysis of radioadaptation induced by low-dose X-rays. Results of the *TK* mutation assay performed under the optimum condition for radioadaptation (Fig. 5) are summarized in the table, and the classification of the isolated TK^- mutants was made by LOH analysis and the results are shown in the histograms (see text).

Table 3. The effect of a priming X-ray exposure on DSB repair (X-ray - X-ray adaptive experiment).

a) NHEJ efficiency in TSCE5 cells

Exp.	Effect of IR (Relative MF*)
1	0.98
2	0.76
3	0.99
Average	0.91

*Relative MF was calculated as MF (X-rays + I-SceI)/MF (I-SceI).

b) HR efficiency in TSCER2 cells

Exp.	Effect of IR (Relative RF*)
1	2.2
2	1.2
3	1.7
Average	1.7

*Relative RF was calculated as RF (X-rays + I-SceI)/RF (I-SceI).

strated that the observed homozygous LOH events were mostly of the crossing-over type.[23] In contrast, the analysis of TK (+/–) revertants observed with our DSB repair assay suggests that HR in this I-SceI system mostly reflects a gene conversion activity, with a relatively small proportion of non-crossing-over events (data not shown). More supporting evidence is required to determine if an enhanced HR activity is reflected by the reduction in homozygous LOH events.

Further applications and perspectives

It is of theoretical and practical importance to estimate human health risks from low-doses of ionizing radiation. One example is the risk for astronauts exposed to space radiation, because the background radiation in space is, at least, more than 100-fold higher than the background level found on earth. Currently, we have the opportunity to study the influence of space radiation in TK6 cells, which were recently brought back to earth after preservation for more than four months, mostly in a frozen state, in the International Space Station. Assuming that the DNA damage caused by space radiation has been accumulated in the frozen cells, such damage could induce mutations when the cells begin to grow again. Furthermore, such damage might have the potential ability to induce radioadaptation and this radioadaptation might be detected as an enhancement in DSB repair in the I-SceI digestion system in the recovered cells.

The following points involved in our I-SceI digestion system merit discussion. Because our I-SceI system does not uncover all NHEJ and HR events, it is difficult to evaluate accurately the extent of DSB repair *via* both HR and NHEJ pathways. For example, our system does not monitor sister-chromatid HR, which is probably the major HR pathway in mammalian cells. Small gene conversion events, which do not extend into the exon 5 region, can also not be detected by this system. Although the I-SceI system may over-estimate the repair efficiency of NHEJ compared with HR, this methodology can still be considered to contribute to elucidating the DSB repair associated with low-dose IR exposure.

Finally, we would like to emphasize that the present evaluation of DSB repair using the I-SceI system, may contribute to our overall understanding of radioadaptation. Other types of studies regarding gene expression, epigenetic changes *etc.*, are also required for a more complete understanding.

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Research Article

Genotoxicity of Acrylamide In Vitro: Acrylamide Is Not Metabolically Activated in Standard In Vitro Systems

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The recent finding that acrylamide (AA), a genotoxic rodent carcinogen, is formed during the frying or baking of a variety of foods raises human health concerns. AA is known to be metabolized by cytochrome P450 2E1 (CYP2E1) to glycidamide (GA), which is responsible for AA's in vivo genotoxicity and probable carcinogenicity. In in-vitro mammalian cell tests, however, AA genotoxicity is not enhanced by rat liver S9 or a human liver microsomal fraction. In an attempt to demonstrate the in vitro expression of AA genotoxicity, we employed *Salmonella* strains and human cell lines that overexpress human CYP2E1. In the *umu* test, however, AA was not genotoxic in the CYP2E1-expressing *Salmonella* strain or its parental strain. Moreover, a transgenic human lymphoblastoid cell line overexpressing CYP2E1 (h2E1v2) and its parental cell line (AHH-1) both showed equally weak cytotoxic and genotoxic responses to high (>1 mM) AA concentrations. The DNA adduct N7-GA-Gua, which is detected in liver following AA treatment in vivo, was not substantially formed in the in vitro system. These results indicate that AA was not metabolically activated to GA in vitro. Thus, AA is not relevantly genotoxic in vitro, although its in vivo genotoxicity was clearly demonstrated. Environ. Mol. Mutagen. 00:000–000, 2010.

Key words: acrylamide; glycydamide; cytochrome P450 2E1 (CYP2E1), in vitro tests; *Salmonella*

INTRODUCTION

Recently, low levels of acrylamide (AA), a synthetic chemical widely used in industry, were detected in a variety of cooked foods [Tareke et al., 2000; Mottram et al., 2002]. It has been proposed that AA forms during frying and baking principally by the Maillard reaction between asparagine residues and glucose [Stadler et al., 2002; Tornqvist, 2005]. This finding raised concerns about a health risk for the general population [Tareke et al., 2002; Rice, 2005].

The International Agency for Research on Cancer classifies AA as 2A, a probable human carcinogen [IARC, 1994]. Because AA clearly induces gene mutations and micronuclei in mice, it could be a genotoxic carcinogen [Cao et al., 1993; Abramsson-Zetterberg, 2003; Manjanatha et al., 2005]. AA is metabolized by cytochrome P450 2E1 (CYP2E1) to glycidamide (GA), which can react with cellular DNA and protein [Sumner et al., 1999; Ghanayem et al., 2005a; Rice, 2005]. Two major GA-DNA adducts, N7-(2-carbamoyl-2-hydroxyethyl)-gua-

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nine (N7-GA-Gua) and N3-(2-carbamoyl-2-hydroxyethyl)-adenine (N3-GA-Ade), have been identified in mice and rats treated with AA or GA [Segerback et al., 1995; Gamboa da Costa et al., 2003; Doerge et al., 2005], with the level of N7-GA-Gua being 100 times as high as the level of N3-GA-Ade in the test organ [Gamboa da Costa et al., 2003]. It is likely that these DNA adducts are responsible for AA's in vivo genotoxicity [Carere, 2006; Ghanayem and Hoffler, 2007]. In our previous study, however, AA did not induce micronuclei in human lymphoblastoid TK6 cells in the presence of rat liver S9, although the genotoxicity of *N*-di-*N*-butylnitrosamine (DBN), which is also metabolized by CYP2E1, was enhanced under the same conditions [Koyama et al., 2006]. Other in vitro genotoxicity studies have also failed to demonstrate the metabolic activation of AA in the presence of S9 [Knaap et al., 1988; Tsuda et al., 1993; Dearfield et al., 1995; Friedman, 2003]. It may be because most S9 preparations have low CYP2E1 activity [Calleman et al., 1990; Hargreaves et al., 1994].

In an attempt to demonstrate the genotoxicity of AA in vitro, we tested the compound using bacteria and mammalian cell lines that express CYP2E1. *S. typhimurium* OY1002/2E1 strain expresses respective human CYP2E1 enzyme and NADPH-cytochrome P450 reductase (reductase), and bacterial *O*-acetyltransferase [Oda et al., 2001]. Using the strain, as well as its parental strain not expressing these enzymes, we conducted an *umu* assay to evaluate induction of cytotoxicity and DNA damage by AA relative to that induced by its metabolite GA. The principle of the *umu* assay is based on the ability of the DNA-damaging agents inducing the *umu* operon. Monitoring the levels of *umu* operon expression enables us to quantitatively detect environmental mutagens [Oda et al., 1985]. In addition, we evaluated the relative mutagenicity of AA vs. GA in assays using transgenic human lymphoblastoid cell lines. Induction of gene mutation at the *TK* locus and of chromosome damage leading to micronucleus (MN) formation were assessed in the h2E1v2 which overexpress human CYP2E1 [Crespi et al., 1993a], vs. its parental cell line, AHH-1. We also investigated the relationship between AA genotoxicity and the formation N7-GA-Gua (derived from GA) in the in vitro mammalian cell system.

MATERIALS AND METHODS

Bacterial Strains, Cell Lines, Chemicals, and Human Liver Microsomal Fraction

For the bacterial tests, we used *umu* strain *S. typhimurium* OY1002/2E1, which expresses human CYP2E1, reductase, and bacterial *O*-acetyltransferase, and its parental strain, *S. typhimurium* TA1535/pSK1002 that does not express these enzymes [Oda et al., 2001].

For the mammalian cell tests, we used human lymphoblastoid cell lines, TK6, AHH-1, and h2E1v2. The TK6 cell line has been described previously [Honma et al., 1997]. The AHH-1 and h2E1v2 cell lines were kindly gifted from Dr. Charles Crespi (BD Bio Sciences, Bedford, MA). AHH-1 is a clonal isolate, derived from RPMI 1788 cells, which was selected for sensitivity to benzo[*a*]pyrene [Crespi and Thilly, 1984]. AHH-1 shows high activity of endogenous CYP1A1. Heterozygosity of AHH-1 cells at thymidine kinase (TK) locus was derived in a two-step selection process utilizing the frameshift mutagen, ICR-191. The AHH-1 cell line was then transfected with plasmids encoding human CYP2E1 enzymes, generating h2E1v2 cell line. AHH-1 expresses CYP1A1 and h2E1v2 expresses both CYP1A1 and CYP2E1 [Crespi et al., 1993a,b].

We purchased AA (CAS No. 79-06-1) and GA (CAS No. 5694-00-8) from Wako Pure Chemical (Tokyo) and dissolved them in phosphate-buffered saline just before use. We purchased *N*-di-*N*-methylnitorosamine (DMN) (CAS No. 62-75-9) from Sigma Aldrich Japan (Tokyo) and dissolved it in DMSO as a positive control for use. We purchased liver S9 prepared from SD rats treated with phenobarbital and 5,6-benzoflavone from the Oriental Yeast (Tokyo). The human liver S9 (HLS-104) was prepared from a human liver sample, which was legally procured from the NDRI (National Disease Research Interchange) in Philadelphia, USA, with permission to use for research purpose only. HLS-104 showed high activity of CYP2E1 [Hakura et al., 2005]. We prepared microsomal fraction from the S9 according to an established procedure [Suzuki et al., 2000]. We prepared the S9- or microsome-mix by mixing 4 ml S9 or microsomal fraction with 2 ml each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP, and 150 mM KCl. CYP2E1 activity of the S9 and microsomal fractions were determined as the activity of chlorzaxazone 6-hydroxylation according to the method of Ikeda et al. [2001].

We grew the cell lines in RPMI1640 medium (Gibco-BRL, Life Technology, Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin, and we maintained the cultures at 10^5-10^6 cells/ml at 37°C in a 5% CO_2 atmosphere with 100% humidity.

umu Assay

The *umu* assay was carried out by the method of Aryal et al. [1999, 2000] with slight modification. Overnight cultures of tester strains were diluted 100-fold with TGlyT medium (1% Bactotryptone, 0.5% NaCl (w/v), 0.2% glycerol (v/v), and 1 μg of tetracycline/ml, 1.0 mM IPTG, 0.5 mMδ-ALA, and 250 ml of trace element mixture/l) [Sandhu et al., 1994]. The culture was incubated for 1 hr at 37°C and then 0.75 ml aliquots of TGA culture (OD_{600}: 0.25–0.3) and human. Induction of the *umuC* gene by HCAs in different strains was determined by measuring cellular β-galactosidase activity, as described by Oda et al. [1985]. Cell toxicity was determined in reaction mixture by measuring the optical density change at 600 nm.

Mammalian Cell Assays Measuring Gene Mutation and Chromosome Damage

We incubated 20-ml aliquots of TK6, AHH-1, or h2E1v2 cell suspensions (5.0×10^5 cells/ml) treated with serially diluted AA, GA, or DMN in the presence or absence of S9 or micorosomes at 37°C for 4 hr, washed them once, resuspended them in fresh medium, and cultured them in new flasks for the MN and TK assays. For TK6 cells, we also seeded cells into the 96-well plates (1.6 cells/well) to determine plating efficiency (PE0).

Forty-eight hours after treating the cells, we prepared the MN test samples as previously reported [Koyama et al., 2006]. At least, 1,000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between nontreated and treated cells were statistically analyzed by Fisher's exact test. The concentration–response relationship was evaluated by the Cochran-Armitage trend test [Matsushima et al., 1999].

We maintained the cultures another 24 hr to allow phenotypic expression prior to plating for determination of the mutant fractions. After the expression time, to isolate the TK deficient mutants, we seeded the cells into 96-well plates in the presence of 3.0 μg/ml trifluorothymidine (TFT).

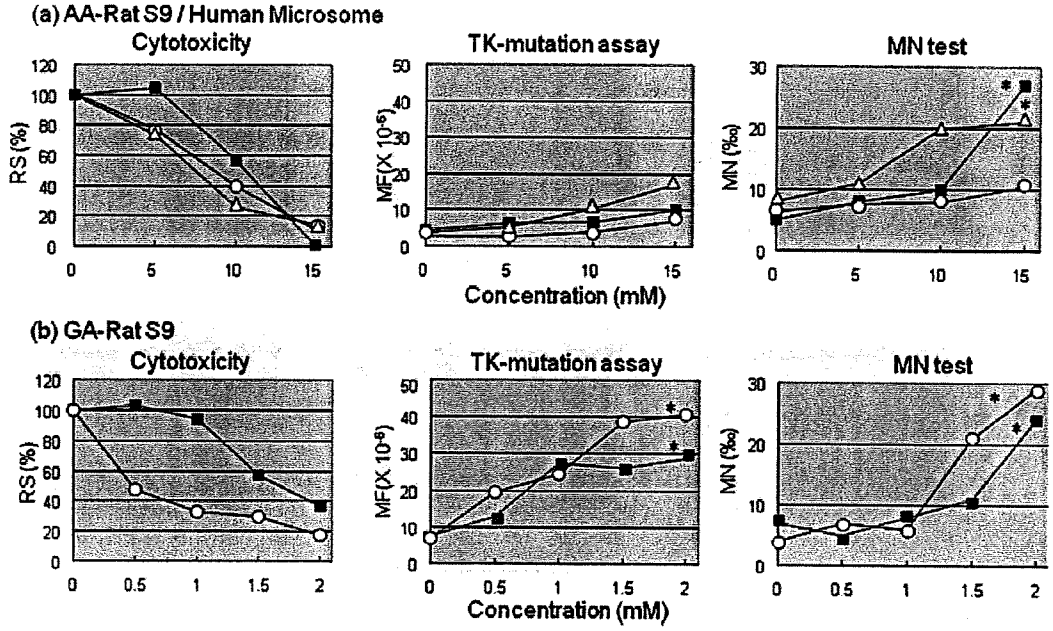

Fig. 1. Cytotoxic (relative survival, RS) and genotoxic (TK and MN assays) responses of TK6 cells treated with AA or GA for 4 hr with or without metabolic activation. (a) TK6 cells were treated with AA without (■) or with (○) rat liver S9 or human microsomes (△). (b) TK6 cells were treated with GA without (■) or with (○) rat liver S9. *$P < 0.05$ (Omori method for TK-mutation assay, trend test for MN assay).

We also seeded cells into the 96-well plates in the absence of TFT to determine plating efficiency (PE3). TK6 cells were seeded at 40,000 cells/well and 1.6 cell/well for TFT and PE plates, respectively. AHH-1 and h2E1v2 cells were seeded at 5,000 cells/well and 3.2 cells/well for TFT and PE plates, respectively. All plates were incubated at 37°C in 5% CO_2 in a humidified incubator. We scored for the colonies in the PE plates at 14th day after plating, and scored for the colonies in the TFT plate on the 28th day after plating. Mutation frequencies were calculated according to the Poisson distribution [Furth et al., 1981]. The data were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson-Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [Omori et al., 2002]. We evaluated cytotoxicity for TK6 by relative survival (RS), which is calculated from plating efficiency (PE0), and for AHH-1 and h2E1v2 by relative suspension growth (RSG), which is calculated from cell growth rate during 3 days expression period.

Western Blot Analysis

A goat polyclonal anti-rat CYP2E1 antibody (Daiichi Pure Chemical, Tokyo) and rabbit anti-rat actin (Sigma, St. Louis, MO) were used as primary antibodies. AP-conjugated secondary antibody (Cappel, Organon Technika Corp., West Chester, PA) was used to detect primary antibody signals.

DNA Adduct Assay

As a standard for LC/MS/MS analysis, N7-GA-Gua and [$^{15}N_5$]-labeled N7-GA-Gua were synthesized as described previously [Gamboa da Costa et al., 2003]. DNA was extracted from the cells by using DNeasy 96 Blood &Tissue Kit (QIAGEN, Düsseldorf) and incubated at 37°C for 48 hr for deprination. An aliquot of the [$^{15}N_5$]-labeled N7-GA-Gua standard was added to each sample and filtered through an ultrafiltration membrane to remove DNA. The eluted-solution was evaporated thoroughly and dissolved in water, and then the solutions were subsequently quantified by LC/MS/MS.

RESULTS

Cytotoxicity and Genotoxicity of AA and GA Under Metabolic Activation

We used human microsomal preparation and phenobarbital- and 5,6-benzoflavone-treated rat liver S9 for metabolic activation. CYP2E1 activity of the human microsomal preparation was more than twice that of the rat liver S9 preparations (2,917 vs. 1,295 pmol/mg/min).

Figure 1 shows the cytotoxicity (RS; relative survival), MN, and *TK*-mutations induced by AA (a) and GA (b) with and without rat liver S9 or human microsomes. Rat liver S9 or human microsomes enhanced cytotoxicity (RS) of AA and GA. On the other hand, AA showed weak genotoxicity only at relatively high concentrations (>10 mM) without S9, but neither activating system enhanced the weak genotoxicty. GA induced TK-mutations dose-dependently from the low concentration (0.5 mM) and induced MN from 1.5 mM both with and without S9. Thus, neither the rat nor human metabolizing system activated AA or inhibited the expression of GA genotoxicity.

umu Assay Using Strains Expressing Human CYP2E1

We used *S. typhimurium* OY1002/2E1 strain to assess the cell toxicity and genotoxicity of AA at exposures up to 10mM (Fig. 2c). We also examined AA and GA with

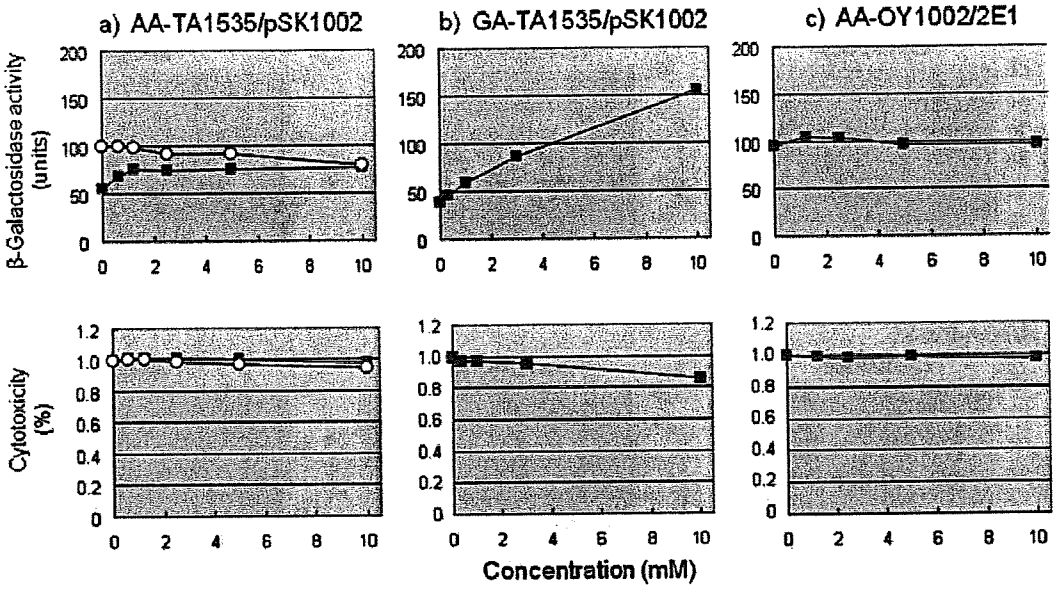

Fig. 2. Induction of *umuC* gene expression and cytotoxic response by AA (a, c) or GA (b) in *S. typhimurium* tester strains TA1535/pSK1002 (a, b) and OY1002/2E1 (c). The *umu* tests were conducted without (■) or with rat S9 (○). β-Galactosidase activity (units) was determined as described in Materials and Methods. Cytotoxic activities are expressed as % optical density change at 600 nm.

or without rat S9 using TA1535/pSK1002 strain. Although GA clearly produced a dose-related increase in response to DNA damage (Fig. 2b), AA elicited no genotoxic or cell toxic response with and without S9 (Fig. 2a). Thus, we could not demonstrate any in vitro genotoxicity of AA in the bacterial system.

Cytotoxic and Genotoxic Responses to AA in Transgenic Cell Lines

Western blot analysis revealed that h2E1v2 accumulated more CYP2E1 than either of its parental cell lines (Fig. 3). Both the h2E1v2 and AHH-1 cells exhibited weak responses (TK-gene mutations and MN) to AA at $\leq$3 mM with little difference in cytotoxicity (RSG, relative suspension growth) (Fig. 4a). h2E1v2 differed from AHH-1, however, in that it showed clear genotoxic and cytotoxic responses (RSG) to DMN, which is a representative substrate for CYP2E1 (Fig. 4b). Thus, the h2E1v2 cell line had CYP2E1 activity but did not activate AA.

DNA Adduct Formation by AA and GA in the Cell Lines

AA induced trace amounts of N7-GA-Gua adduct in TK6 cells (with and without S9) (Fig. 5a) and in AHH-1 and h2E1v2 cells (Fig. 5b). GA, on the other hand, induced a substantial number of N7-GA-Gua adducts in TK6 cells (Fig. 5c). These results suggest that the expression of genotoxicity may be dependent on N7-GA-Gua adduct formation, and the in vitro metabolic activation system did not metabolize AA into GA.

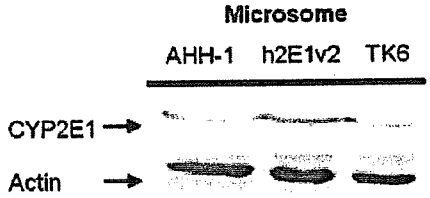

Fig. 3. Western blot analysis of CYP2E1 in AHH-1, h2E1v2, and TK6 cells. Equal amount of materials were loaded for each sample. CYP2E1 protein was stained with the anti-CYP2E1 antibody. Actin was used as a loading control.

DISCUSSION

A large number of studies about the in vitro genotoxicity of AA have been reported [Dearfield et al., 1995; Besaratinia and Pfeifer, 2005]. AA was negative in Ames assay in both the presence and absence of S9 [Zeiger et al., 1987; Knaap et al., 1988; Tsuda et al., 1993]. In mammalian cell assays, cytogenetic tests such as chromosome aberration test and sister chromatid exchange tests were positive [Sofuni et al., 1985; Tsuda et al., 1993]. AA also induced *Tk* mutation in the MLA but did not induce *Hprt* mutation in V79 cells [Moore et al., 1987;

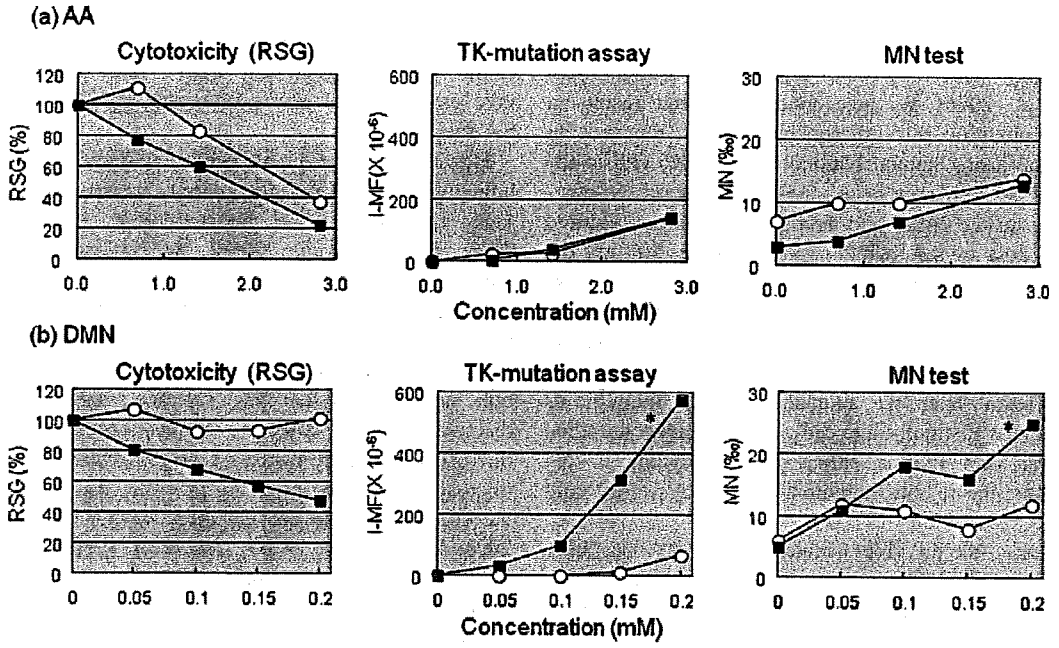

Fig. 4. Cytotoxic (relative suspension growth, RSG) and genotoxic (TK assay and MN test) responses of AHH-1 (○) or h2E1v2 (■) cells treated with AA or DMN for 4 hr. I-MF means induced mutation fraction, in which back ground mutation frequency is subtracted. *$P < 0.05$ (Omori method for TK-mutation assay, trend test for MN assay).

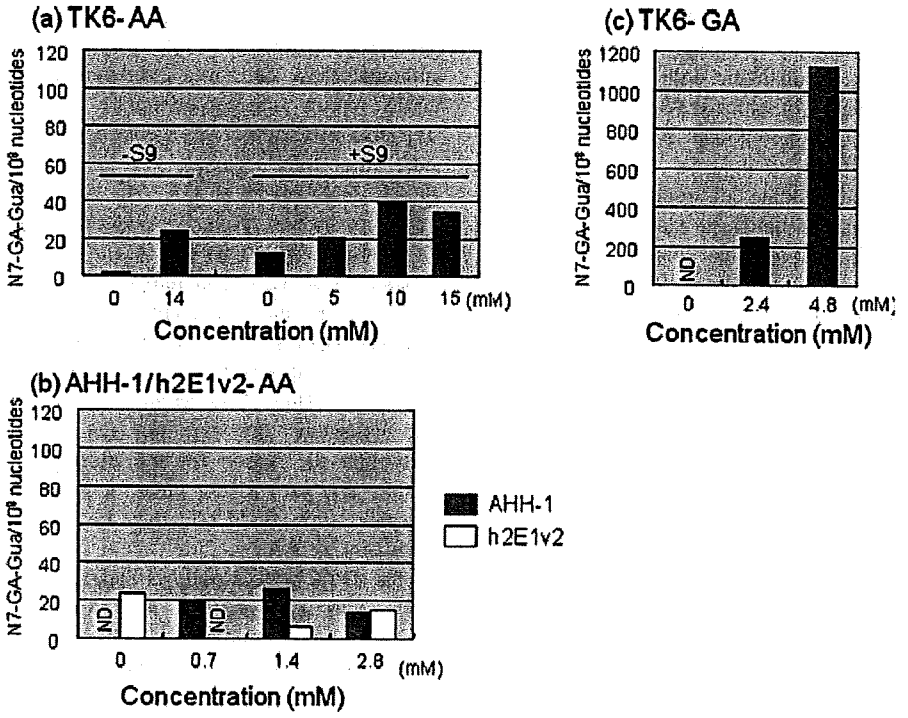

Fig. 5. Levels of N7-GA-Gua adduct in TK6 (a, c), AHH-1 (b), or h2E1v2 (b) cells treated with AA (a, b) or GA (c) for 4 hr at different concentrations. Data are expressed as the number of adducts in 10^8 nucleotides.

Knaap et al., 1988; Tsuda et al., 1993; Baum et al., 2005; Mei et al., 2008], and produced negative results in the Comet assay with V79 cells and human lymphocytes [Baum et al., 2005]. We also obtained positive results in *TK* gene mutation and micronuclei assays, but not in Comet assay using human lymphoblastoid TK6 cell in the absence of S9 [Koyama et al., 2006]. To obtain the positive results in the MLA and TK6 cells, however required very high dose of AA, which was sometimes beyond the top dose of the OECD testing guideline (>10 mM) [Koyama et al., 2006; Mei et al., 2008]. The spectrum of AA-induced *TK* mutations in TK6 and *cII* mutations in Big Blue® mouse embryonic fibroblasts were not significantly different from the spontaneous one, although its metabolite GA distinctly induced a specific point mutation [Besaratinia and Pfeifer, 2003, 2004; Koyama et al., 2006]. Thus, the in vitro genotoxicity of AA is still controversial.

In contrast, the in vivo genotoxicity of AA has been clearly demonstrated by various rodent genotoxicity tests including micronuclei tests in peripheral blood [Cao et al., 1993; Abramsson-Zetterberg, 2003; Manjanatha et al., 2005], transgenic gene mutation in liver [Manjanatha et al., 2005], and Comet assay in various organs [Ghanayem et al., 2005b]. AA has also proven to be genotoxic to germ cells [Dearfield et al., 1995]. AA induced micronuclei in mice spermatids, and heritable chromosome translocations and specific locus mutations in postomeiotic sperm and spermatogonia [Lahdetie et al., 1994; Xiao and Tates, 1994]. AA also elevated the frequency of dominant lethal mutations probably accompanying with chromosome aberrations leading to death of embryo [Shelby et al., 1987; Adler et al., 1994]. The International Agency for Research on Cancer (IARC) classified it as 2A, a probable human carcinogen based on finding of rodent carcinogenicity [IARC, 1994]. AA caused tumors in various organs including mammary gland, peritesticular mesothelium, thyroid, and central nervous system [Carere, 2006], although the AA-inducing genotoxicity in these organs have not been demonstrated.

AA is metabolized either via direct glutathione conjugation followed by excretion of mercapturic acid or via oxidative pathways catalyzed by CYP2E1 to yield GA [Calleman et al., 1990; Wu et al., 1993; Sumner et al., 1999]. GA reacts quickly with DNA, mainly forming N7-GA-Gua adduct. Genotoxicity of GA has been demonstrated in vitro and in vivo. In contrast to AA, GA is positive in most genotoxicity tests [Hashimoto and Tanii, 1985; Dearfield et al., 1995; Besaratinia and Pfeifer, 2004; Baum et al., 2005; Koyama et al., 2006]. Manjanatha et al. [2005] demonstrated in transgenic Big Blue® mice that both AA and GA induces endogenous *Hprt* and transgenic *cII* mutation at same level, and also produced similar mutational spectra. The predominant type of mutations observed in these two systems was G:C to T:A transversion, which is presumably derived from N7-GA-Gua [Besaratinia and Pfeifer, 2005]. The in vivo results with transgenic Big Blue® mice indicate that in vivo expression of AA genotoxicity is mediated via its GA metabolite.

However, no one has succeeded in demonstrating metabolically activated AA genotoxicity in vitro [Knaap et al., 1988; Tsuda et al., 1993; Dearfield et al., 1995; Friedman, 2003; Emmert et al., 2006]. In this study, we used induced rat liver S9 and human microsomal fraction for the metabolic activation. Although they have high CYP2E1 activity, the AA-inducing genotoxicity was never influenced by the presence of the exogenous metabolic activation system (Fig. 1a). We assumed that GA, a reactive epoxide, could be rapidly inactivated through microsomal epoxide hydrolase or glutathione in any S9 or microsomal fraction resulting in either the metabolism or the conjugation and detoxification of GA [Sumner et al., 2003; Decker et al., 2009]. However, presence of rat S9 did not prevent GA from inducing *TK*-mutation and micronuclei.

The *umu* assay could not detect the genotoxicity of AA even by the strain (Fig. 2). Emmert et al. [2006] also failed to demonstrate the mutagenicity of AA in the Ames test using the metabolically competent *S. typhimurium strain* YG7108pinERb$_5$ that expresses CYP2E1. In mammalian cell system, such as the human lymphoblastoid cell line, h2E1v2 overexpressing human CYP2E1 did not show different response in *TK*-gene mutation and MN induction compared to its parental cell line, AHH-1, although these cell lines exhibited distinct difference to DMN, which is a representative substrate for CYP2E1. We also investigated the genotoxicity of AA in h2E1v2 cells after long exposure (24 hr), because AA may be slowly metabolized to GA. The result was also negative (data not shown). Thus, we could not obtain any evidence of in vitro genotoxicity of AA *via* metabolic activation.

Glatt et al. [2005] developed a Chinese hamster V79-derived cell line that stably expresses human CYP2E1 and sulphotransferase (SULT), and applied it to investigate sister chromatid exchanges (SCE) induced by some chemicals. They demonstrated that AA induced SCE in the transgenic cell line but not in the parental line. Although the reason for the discrepancy between their results and ours is not clear, it is possible that another enzyme, such as SULT, may be involved in metabolic activation of AA.

The DNA adduct analysis clearly revealed that h2E1v2 cells does not generate N7-GA-Gua adduct in vitro. Because exposure of human cells to GA results in significant accumulation of N7-GA-Gua adduct, but DNA adduct analysis following exposure of h2E1v2 with AA does not generate N7-GA-Gua adduct in vitro, lead one a conclusion that the presence of CYP2E1 alone is not enough to metabolize AA to GA in mammalian cells. The

DNA adduct analysis also strongly supports a hypothesis that GA contribute to its genotoxicity by forming N7-GA-Gua adduct. Interestingly, very small amount of N7-GA-Gua adduct was generated in TK6 cells in a dose-dependent manner regardless of the presence of S9 (Fig. 5a). TK6 cells themselves may have an enzymatic activity to metabolize AA to GA, although its activity must be extremely low. Ghanayem et al. [2005b] showed that AA was not mutagenic or genotoxic in CYP2E1-null mice. Intraperitoneal injection of AA (25, 50 mg/kg) by once daily for 5 days induced micronuclei in erythrocyte and DNA damage assessed by Comet assay in leukocyte and liver cells of wild-type, but not in the CYP2E1-null mice. The plasma concentration of AA in the CYP2E1-null mice was 115-times higher than in the wild-type mice, while the GA concentration in the CYP2E1-null mice was negligible compared to that in the wild-type mice [Ghanayem et al., 2000]. Ghanayem et al. [2005c] also demonstrated that AA produces dominant lethal in mice that express CYP2E1, but not in mice that do not express CYP2E1, indicating that induction of germ cell mutations by AA in mice in vivo is also dependent upon CYP2E1 metabolism. These results clearly suggest that CYP2E1 is the principal enzyme responsible for the metabolism of AA to GA in vivo.

In conclusion, AA could not be metabolized to GA by in vitro metabolic activation system commonly used in genotoxicity tests. In vivo, on the other hand, GA is apparently responsible for AA-inducing genotoxicity. Although AA may exhibit genotoxicity in in vitro mammalian cells at high concentrations, its positive response is not relevant for its major genotoxicity. AA could be classified into in vivo specific genotoxic chemical.

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