むしろ幼若ラットの方が増加率は低かった。

精巣に関しては小核、コメット試験を実施した（図４、図５）。両試験において幼若ラットで用量依存的な反応性と、100、200ppm 投与群での有意な誘発を認めた。また、小核に関しては幼若、成熟ラット間で顕著な反応性の違いが観察された。

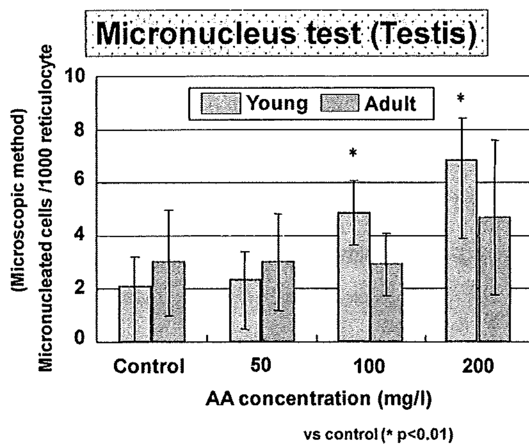

図４

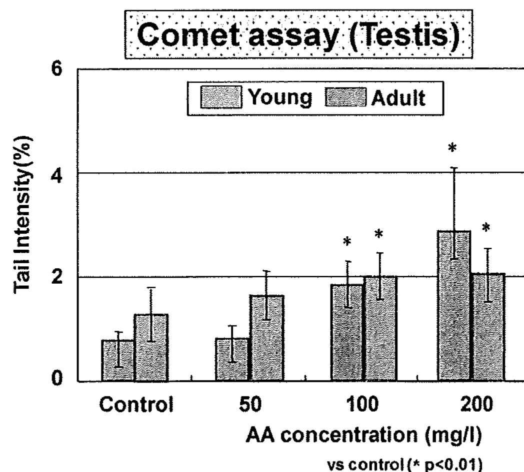

図５

甲状腺、乳腺、肝臓、精巣における AA の主たる DNA アダクトである *N*7-GA-Gua を LC/MS/MS により測定した。全ての組織において DNA アダクトは用量依存的に増加したが、甲状腺、乳腺、肝臓においては幼若、成熟ラット間で差は認められなかった。一方、精巣におけるアダクト量は幼若ラットで顕著に高かった（図６）。

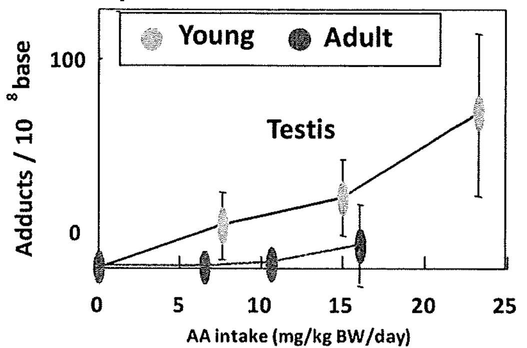

図６

D．考　察

In vitro において AA と GA の遺伝毒性について比較したところ、AA はほとんど in vitro では毒性を示さなかったのに対して、GA の遺伝毒性は強く、主として点突然変異を主体とする DNA 損傷作用を持つことが報告されている。このことから、AA の毒性の本体はその代謝物である GA であると結論した。AA は CYP2E1 により、エポキシ環をもつ GA に変換され、強い遺伝毒性物質に変わるが予想される。しかしながら、これまでの我々の研究では in vitro の試験系で AA を GA に代謝活性化する系を作ることはできなかった。我々は AA の遺伝毒性は in vivo の系のみで正当に評価されると結論づけた。

これまで AA の in vivo の遺伝毒性に関しては Manjanatha らによるトランスジェニック BigBlue マウスを用いた報告がある。彼らは雄雌ラットそれぞれに 100、500ppm の飲水で 4 週間日間投与したところ、肝臓での突然変異が有意に増加し、GC>TA のトラ

ンスバージョンが主な変異であることを報告した。

我々の実験は Manjanatha ら実験と比較し、低い濃度での検討を行った(50, 100, 200ppm)。尚、200ppm の飲水投与での AA 摂取量は 20mg/kg/day に相当し、これはこれまでの AA での毒性試験の 1/10 程度の量に相当する。全ての試験動物について生育に伴う顕著な体重の抑制、飲水量の変化は認められなかった。また、28 日後の剖検においても顕著な病理的異常は認められなかった。

骨髄、末梢血に関しては、顕著な小核誘発は認められなかった。Manjanatha らの報告でも、100ppm では骨髄小核の誘発は認められていない。統計的には幼若ラットの骨髄、両軍ラットの末梢血で有意差が認められた。また、pig-A 突然変異も最高用量において、幼若ラットで有意差が認められた。pig-A 突然変異試験は末梢血を用いた新規遺伝毒性試験であり、表面抗原である PIG アンカータンパク質の欠損細胞を突然変異体としてフローサイトメーターで分画する試験である。小核試験、コメット試験のような一過性の遺伝毒性と異なり、変異体は長期間体内に留まるため、本研究のような低用量、長期間暴露の慢性の試験系に適すると考えられている。今回の試験での Pig-A の遺伝毒性の発現は小核と同様のパターンを示した。すなわち、幼若ラットのみで弱い反応性示した。飲水投与による慢性暴露が血球細胞に遺伝子突然変異をもたらすことが明らかとなった。しかしながら、幼若、成熟ラットでの差は顕著ではなかった。同様のことは肝臓についても言える。造血系、肝臓に対して AA は弱い遺伝毒性を示すが、幼若ラットで特に強い影響があるとは考えにくい。以前我々は gpt ラットを用い 20-80ppm の AA での飲水投与実験を行い、肝臓での突然変異を検討したが、幼若、成熟ラットとも突然変異の誘発は認められなかった。この結果は先の Manjanatha らの報告と矛盾するが、1)種差、2)投与量の違いが考えられる。いずれにせよ、200ppm 程度の AA の肝臓に対する遺伝毒性はそれほど強くはないと考えられた。

精巣に関しては、小核試験、コメット試験で成熟、幼若ラットと共に用量依存性の増加が観察された。また、この増加は幼若ラットで顕著であった。DNA アダクトの結果はこのことを裏付けるものである。GA は N7-G-GA、N3-A-GA、N1-A-GA の３種類の DNA アダクトを生成することが知られているが、N7-G-GA が全体の 90%以上を占めるため、今回、このアダクトにのみ注目して測定した。精巣での N7-G-GA は幼若、成熟ラットともに用量依存的に増加し、特に幼若ラットでは最高用量（200ppm）で、成熟ラットと比較して、も 10 倍以上高いアダクトの生成が観察された。

これまで、精巣には AA のアダクトが蓄積しやすく、その原因としてプロタミンとの結合が考えられている。また、その約５％は DNA ともアダクトを形成する。AA は精巣細胞に強い遺伝毒性を示し、転座型の染色体異常を示すこと、低い濃度でも優性致死試験で陽性を示すことが知られている。このように AA は特に生殖細胞に遺伝毒性を示し、それがアダクトの生成と相関するものと考えられる。さらに今回、我々の実験ではこの傾向が幼若ラットで顕著に現れることが示された。この原因については不

明であり、今後の解明が待たれるが、少なくともAAの生殖細胞に対する遺伝毒性感受性には年齢が関係しており、幼年期でのAAの過剰摂取に関しては、注意が必要であると考えられる。

E．結　論

50、100、200ppmのアクリルアミドを飲水で成熟、および幼若ラットに投与し、各種遺伝毒性と、DNAアダクト形成を比較し、ライフステージの違いによるAAの遺伝毒性感受性の差を検討した。多くの組織で、ライフステージの違いよる差は認められなかったのに対して、精巣ではDNA損傷、小核の誘発が幼若ラットで顕著であった。また、それに対応したDNAアダクト量の増加も観察された。特にアダクト量は、幼若ラットでは成熟ラットに比較し、10倍以上もの蓄積を認めた。本来、AAには生殖細胞に対する強い遺伝毒性が報告されている。今後、ライフステージを勘案した生殖細胞に対するAAの遺伝毒性メカニズムを解明すると同時に、低年齢層に対するAAの遺伝毒性リスクを考察する。

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G．知的所有権の取得状況

なし

別添５

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Letter

Increased H-*ras* mutation frequency in mammary tumors of rats initiated with *N*-methyl-*N*-nitrosourea (MNU) and treated with acrylamide

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ABSTRACT — We recently demonstrated the incidence and multiplicity of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary tumors to be increased by administration of acrylamide (AA) in post-initiation in rats. In the present study, to clarify the mechanisms of enhancement, H-*ras* gene mutations in mammary tumors induced in MNU-initiated rats with or without subsequent AA administration were investigated. Frequencies of mutations in codon 12 from GGA to GAA were significantly ($p < 0.05$) higher in rats with AA administration (82%, 23 out of 28 tumors) as compared to those without AA (50%, 9 out of 18 tumors), but the latency and volume of H-*ras* mutation-harboring tumors were similar to those of the mutation-lacking tumors. No mutations in codons 13 or 61 were detected in either treatment groups. The results thus indicate that H-*ras* gene mutations in codon 12 play a pivotal role in initiation of carcinogenesis and it appears possible that AA administration may selectively co-stimulate and/or maintain initiated cells via other genomic or non-genomic events in MNU-treated rats.

Key words: Acrylamide, Mammary tumors, H-*ras* gene, Rat

INTRODUCTION

Acrylamide (AA) has found many commercial and industrial applications, e.g. in water treatment, soil stabilization, paper making, and for electrophoresis gels in biotechnology laboratories (IARC, 1994). In addition, it has recently been found in fried and/or baked carbohydrate-rich foods at various concentrations, resulting in public health concerns (Rosén and Hellenäs, 2002; Tareke *et al.*, 2002), given its classification as probably carcinogenic in humans (IARC, 1994). In rat long-term studies, the incidences of multi-organ tumors including scrotal mesotheliomas in males, mammary gland tumors in females and thyroid follicular cell tumors in both males and females were increased by AA administration in the drinking water at concentrations required to provide a dose of 0.5-2.0 mg/kg body weight/day to males or 1.0-2.0 mg/kg body weight/day to females (Friedman *et al.*, 1995; Johnson *et al.*, 1986). Six oral administrations of AA at doses ranging from 12.5 to 50 mg/kg over 2 weeks also induced squamous cell papillomas and carcinomas in the skin of Swiss-ICR and Sencar mice in the presence of the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (Bull *et al.*, 1984a, 1984b) and lung tumors were increased in A/J mice which received i.p. injections at 1 to 60 mg/kg body weight or p.o. administration at 1.05 to 42 mg/kg body weight, 3 times /week for 8 weeks (Bull *et al.*, 1984a). In addition, we recently reported that the incidence and multiplicity of mammary tumors were increased by AA-administration at calculated average intakes of 2.3 and 5.0 mg/kg body weight/day in drinking water for 30 weeks in rats initiated with *N*-methyl-*N*-nitrosourea (MNU) (Imai *et al.*, 2005). However, the precise mechanisms underlying such carcinogenic effects of AA remain uncertain.

MNU-induced mammary carcinomas often carry a specific G to A transition mutation at the second base

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of codon 12 of H-*ras* and it has been proposed that this mutation contributes to the initiation of carcinogenesis (Lu *et al.*, 1991; Sukumar *et al.*, 1995; Zarbl *et al.*, 1985). Its presence in normal-appearing mammary gland and early preneoplastic lesions of MNU-treated rats is in line with a critical role in the early stages of MNU-induced carcinogenesis (Korkola and Archer, 1999; Kumar *et al.*, 1990; Sakai and Ogawa, 1991). H-*ras* codon 12 is also susceptible to induction of mutations by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Ushijima *et al.*, 1994; Yu and Snyderwine, 2002), while codons 13 and 61 often exhibit PhIP and 7,12-dimethylbenz[*a*]anthracene (DMBA)-DNA adduct-induced mutations, respectively (Kito *et al.*, 1996; Ushijima *et al.*, 1994; Yu and Snyderwine, 2002). In the present study, to cast light on mechanisms AA enhancement of rat mammary carcinogenesis, H-*ras* mutations in mammary tumors induced in MNU-initiated rats with and without subsequent AA administration were investigated using a direct sequencing technique. In particular, this mutation analysis was undertaken with a view to confirm whether any AA-specific H-*ras* mutation patterns could be detected or not in mammary tumors of MNU-initiated rats followed by AA administration and whether the mutation frequency of a predominant G to A transition at H-*ras* codon 12 would be altered by AA administration or not via other genomic or non-genomic events in MNU-induced rat mammary tumors.

MATERIALS AND METHODS

Mammary tumor samples used in the present study were obtained from the experiment described in our previous report (Imai *et al.*, 2005). Briefly, a total of 60 female Sprague-Dawley rats (Crj:CD(SD)IGS, Charles River Japan Inc., Kanagawa, Japan) were treated with a single intraperitoneal injection of MNU at a dose of 50 mg/kg body weight and then given free access to drinking water containing 0, 20 or 40 ppm AA for 30 weeks. During the AA treatment period, thoracic and abdominal mammary gland tumors apparent on palpation were recorded weekly. Palpable tumor volumes were calculated as (length) x (depth) x (height) x 0.52. At the end of the experimental period, all rats were necropsied and all subcutaneous tumors were collected and their sizes measured for volume calculation in the same manner as for palpable tumors. Incidences and multiplicities of tumors were increased in an AA-dose-dependent manner. Particularly, the incidence at 40 ppm was significantly ($p < 0.05$) elevated as compared to the 0 ppm group. Histopathologically, all the tumors were diagnosed as adenocarcinoma except for one case of fibroadenoma in the 40 ppm group. In the present study, 18 and 28 randomly selected frozen samples dissected from mammary tumors diagnosed as adenocarcinoma in the 0 and 40 ppm AA groups, respectively, were prepared for genomic DNA extraction using a standard phenol-chloroform protocol for mutation analysis. For direct sequencing analysis of H-*ras* mutations, exon 1 of the gene was amplified with the primers 5'-GCAGTCTCAAGTGGCTAGGG-3' and 5'-TGGGATCATACTCGTCCACA-3', and exon 2 with the primers 5'-AGGACCCTTAAGCTGTGTTC-3' and 5'-CCCGCATGGCACTATACTCT-3' at annealing temperatures of 62°C and 64°C, respectively, both with 30 cycles of polymerase chain reaction (PCR). The resulting amplicons were prepared for nucleotide sequencing by enzymatic removal of unused deoxyribonucleoside triphosphate (dNTP) and primers. An enzyme preparation (ExoSAP-IT; GE Healthcare Bio-Sciences, Piscataway, NJ, USA; 1 µl) was added directly to 5 µl of the PCR product followed by incubation at 37°C for 15 min and inactivation of the enzyme by heating to 80°C for 15 min. The sequencing was performed following the manufacturer's protocol with a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare Bio-Sciences). Briefly, 3 µl of purified PCR product was mixed with 4 µl of Ready Reaction Premix, 4 µl of DYEnamic ET Terminator Dilution Buffer (GE Healthcare Bio-Sciences), 0.5 µl of 20 µM forward primer solution and 8.5 µl of dH_2O. This mixture was thermal cycled 25 times at 95°C for 20 sec, 50°C for 15 sec and 60°C for 1 min. After the clean-up procedure for free nucleotides with AutoSeq G-50 purification columns (GE Healthcare Bio-Sciences) sequences were analyzed with a DNA sequencer ABI 310 (Applied Biosystems, Foster City, CA, USA). In addition, corresponding paraffin sections of mammary tumors to the frozen samples for H-*ras* mutation analysis were used for counting of Ki-67 positivities, as a marker of cell proliferation. For the immunohistochemistry, an anti-Ki-67 antigen monoclonal antibody (clone MIB-5; DAKO cytomation, Glostrup, Denmark) and the streptavidin-biotin-peroxidase complex method (StreptABComplex, DAKO cytomation) were used.

Statistical analysis

The multiplicity and volume data of mammary gland tumors and Ki-67 positive indices were analyzed using the Student's or Welch's *t*-test following the *F*-test. For incidence and H-*ras* codon 12 mutation frequency values of mammary tumors, the Fisher's exact probability test was applied.

RESULTS

Data for incidence, latency, multiplicity and/or volume of mammary tumors in the animal study selected for the H-*ras* mutation analysis in MNU-initiated rats with or without subsequent AA administration are shown in Table 1. The average latency and volume of tumor samples selected for the present analysis of H-*ras* mutations were similar to those of the total samples overall in the animal study.

Direct sequencing of mammary tumor samples diagnosed as adenocarcinoma bearing the H-*ras* codon 12 mutation (GGA to GAA) revealed a high extra-signal of A in the second base (Fig. 1). The frequency of the H-*ras* mutation in codon 12 in tumor samples of rats treated with MNU followed by 40 ppm AA administration was 82% (23 out of 28 tumors) and significantly ($p < 0.05$) higher than that in tumor after treatment with MNU alone (50%, 9 out of 18), but the latency and volume of H-*ras* mutation-harboring tumors were similar to those of the mutation-lacking tumors (Table 1). No mutations in codon 13 or 61 were detected in any of the samples examined.

Ki-67 positive indices in mammary tumors of rats treated with MNU followed by 40 ppm AA administration appeared to be lower than those after treatment with MNU alone albeit without statistical significance (Table 2). There was no significant difference in Ki-67 positivities between the tumors with and without the H-*ras* mutation.

DISCUSSION

Frequencies of mutations in codon 12 from GGA to GAA were significantly higher in rats with AA administration as compared to those without AA, but the latency and volume of H-*ras* mutation-harboring tumors were similar to those of the mutation-lacking tumors. Ki-67 immunohistochemistry revealed that cell proliferation in mammary tumor was not activated by AA administration. In addition, there was no significant difference in Ki-67 positivities between the tumors with and without the H-*ras* mutation. These findings suggest that while the H-*ras* gene mutation in codon 12 may play a pivotal role in the initiation of carcinogenesis, AA administration may not be directly related to tumor cell proliferation-stimulating activity, but rather co-selectively stimulate and/or maintain cells containing the H-*ras* mutation in codon 12 so that mammary carcinogenesis is enhanced.

AA did not show mutagenic activity in *Salmonella*/microsome test systems and mammalian cells (Hashimoto and Tanii, 1985; Tsuda *et al.*, 1993), but chromosomal aberrations, sister chromatid exchange, unscheduled DNA synthesis and morphological transformation were clearly evident in treated mammalian cell lines (Banerjee

Table 1. Data for incidence, multiplicity, volume and latency of mammary tumors and frequency of H-*ras* codon 12 mutations in randomly selected tumor samples of rats treated with MNU followed by AA administration

Treatment	MNU + AA 40 ppm		MNU only	
No. of animals used in the experiment	20		20	
Mammary tumors				
Incidence [a]	16 (80) *		10 (50)	
Latency (weeks after MNU-initiation) [b]	21.30 ± 7.15		21.30 ± 6.63	
Multiplicity (no./rat) [a, b]	2.10 ± 2.53		1.00 ± 1.34	
Volume (cm³) [a, b]	4.57 ± 6.65		4.78 ± 5.87	
No. of selected tumor samples for H-*ras* mutation analysis	28		18	
Latency (weeks after MNU-initiation) [b]	21.93 ± 5.66		21.93 ± 5.66	
Volume (cm³) [b]	4.87 ± 6.84		4.68 ± 5.95	
H-*ras* mutation in codon 12	present	absent	present	absent
Mutation frequency	23 (82) #	5 (18)	9 (50)	9 (50)
Latency (weeks after MNU-initiation) [b]	22.00 ± 5.79	23.20 ± 4.66	22.00 ± 5.15	20.67 ± 0.83
Volume (cm³) [b]	5.06 ± 7.11	4.00 ± 7.11	4.90 ± 5.51	4.47 ± 6.69

*, $P < 0.05$ vs. the MNU only group.
#, $P < 0.05$ vs. the MNU only group with H-*ras* codon 12 mutation.
a, data from the experiment described in our previous report (Imai *et al.* 2005).
b, data are mean ± S.D. values.
AA, acrylamide; MNU, *N*-methyl-*N*-nitrosourea.
Parentheses, %.

and Segal, 1986; Knaap *et al.*, 1988; Park *et al.*, 2002; Sega *et al.*, 1990; Tsuda *et al.*, 1993). Thus AA may function through some alterative mechanisms other than direct DNA interaction. On the other hand, an epoxide metabolite of AA, glycidamide (GA) is known to be readily reactive toward DNA (Dearfield *et al.*, 1995) and *in vivo* oral or intraperitoneal dosing of AA to mice and rats may induce N7-(2-carbamoyl-2-hydroxyethyl)-guanine (N7-GA-Gua), a GA derived DNA adduct in liver, kidney, lung, brain or testis (Gamboa da Costa *et al.*, 2003; Segerbäck *et al.*, 1995). This N7-GA-Gua was found to be approximately 100-fold more extensive than a separate GA-derived DNA adduct, 3-(2-carbamoyl-2-hydroxyethyl)-adenine (N3-GA-Ade), in liver, lung and kidney (Gamboa da Costa *et al.*, 2003), suggesting that AA-metabolites demonstrate higher binding affinity to guanine than adenine bases. In addition, the predominant types of mutations in the liver *cII* gene from AA- or GA-treated mice were G to T transversions (Manjanatha *et al.*, 2006). On the other hand, MNU-induced mammary carcinomas have been reported to harbor a predominant G to A transition mutation at the second base of H-*ras* codon 12 and this mutation is considered to contribute to the initiation of carcinogensis (Lu *et al.*, 1991; Sukumar *et al.*, 1995; Zarbl *et al.*, 1985). The results thus indicate that the increased frequency of G to A transition in codon 12 with no additional mutations in other sites of H-*ras* gene in tumors in the present study may not be related to any direct DNA interaction of AA and GA.

As another possible hypothesis, AA and/or GA could induce other genomic or non-genomic events that would impact on cells having H-*ras* codon 12 mutations, e.g. oxidative stress-induced DNA damage or pericellular environmental change. Both AA and GA have been shown to conjugate with glutathione (Sumner *et al.*, 1999), which may lead to depletion of cellular GSH stores and resulting oxidative stress. Park *et al.* (2002) reported that co-incubation of AA with N-acetyl-L-cysteine, a SH group donor, resulted in reduction of AA-induced morphological transformation in Syrian hamster embryo (SHE) cells. Recent results showed that GSH supplementation may protect against DMBA-induced mammary carcinogenesis in female Sprague Dawley rats (Anbuselvam *et al.*, 2007; Padmavathi *et al.*, 2006). AA can bind to cysteine SH of proteins by alkylation (Friedman, 2003; Sega, 1991), and thus could conceivably exert modifying effects on mammary carcinogenesis. As another possibility, hormonal activity or endocrine disruption may be involved in enhancement of mammary carcinogenesis by AA, since the parent compound and GA can both stimulate steroid hormone production (Clement *et al.*, 2007; Shiver *et al.*, 1992). Although estradiol and progesterone treatment in aged rats can promote MNU-induced carcinogenesis (Tsukamoto *et al.*, 2007), in younger individuals the opposite effects are exerted without alteration of the H-*ras* mutation frequency (Swanson and Christov, 2003). In the present study, MNU-induced mammary carcinogenesis was enhanced by AA in relatively early life stages and the H-*ras* mutation frequency in the tumors

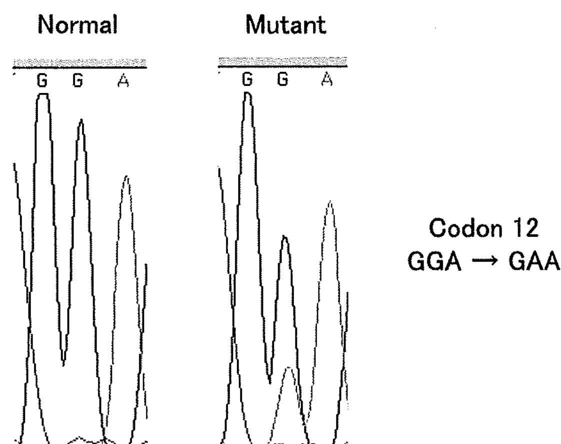

Fig. 1. Results of direct sequencing analysis of codon 12 of H-*ras* in MNU-induced mammary tumors. The mutant sample demonstrates an extra-signal for A in the second base indicating the presence of a mutation.

Table 2. Ki-67 indices in mammary tumors with or without H-*ras* codon 12 mutations of rats treated with MNU followed by AA administration

Treatment	MNU + AA 40 ppm		MNU only	
H-*ras* mutation in codon 12	present	absent	present	absent
No. of tumor sample for Ki-67 index	23	5	9	9
Ki-67 positive index (%) [a]	17.24 ± 6.76	15.82 ± 9.7	22.45 ± 8.25	19.86 ± 7.81

[a], data are mean ± S.D. values.
AA, acrylamide; MNU, *N*-methyl-*N*-nitrosourea.

was significantly increased, suggesting hormonal actions for mammary carcinogenesis by AA being still unclear. Further studies are needed to clarify this point.

In summary, the present examination of mutation frequencies in the H-*ras* gene in mammary tumors of rats treated with or without 40 ppm AA in the drinking water following MNU-initiation showed significant increase in GGA to GAA transitions affecting codon 12 by AA administration. However, the latency and volume of H-*ras* mutation harboring tumors were similar to those of the mutation lacking tumors. Further detailed examination of apoptosis inhibiting or hormonal activities of AA/GA should help further delineate the mechanisms of AA-related mammary carcinogenicity.

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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. © 2010 Wiley-Liss, Inc.

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 *umu*C 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).

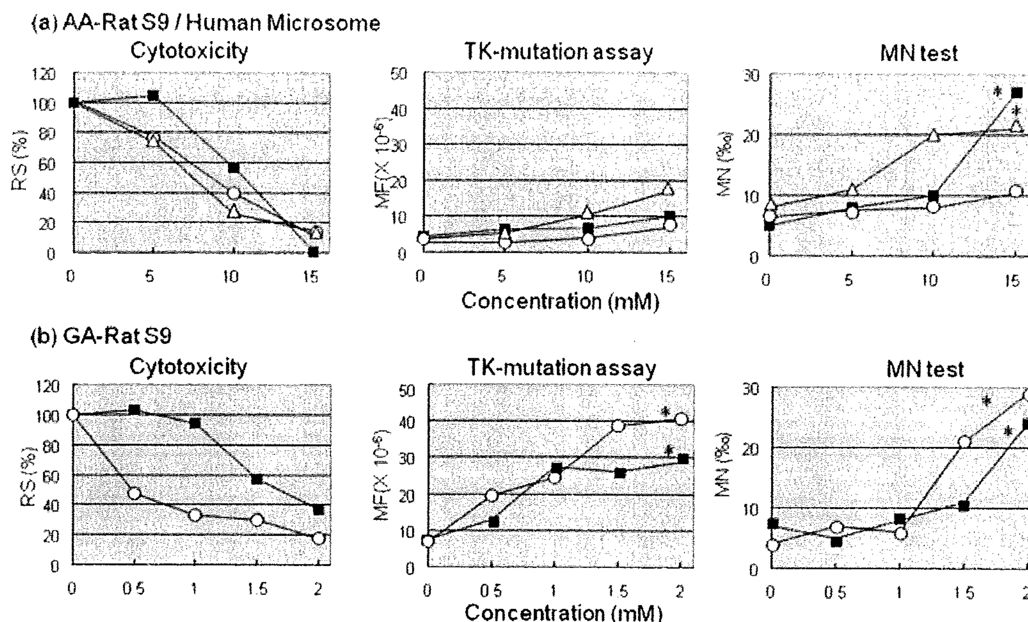

Fig. 1. Cytotoxic (relative survival, RS) and genotoxic (TK and MN assays) responses of TK6 cells treated with AA or GA for 4 hr with or without metabolic activation. (**a**) TK6 cells were treated with AA without (■) or with (○) rat liver S9 or human microsomes (△). (**b**) TK6 cells were treated with 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

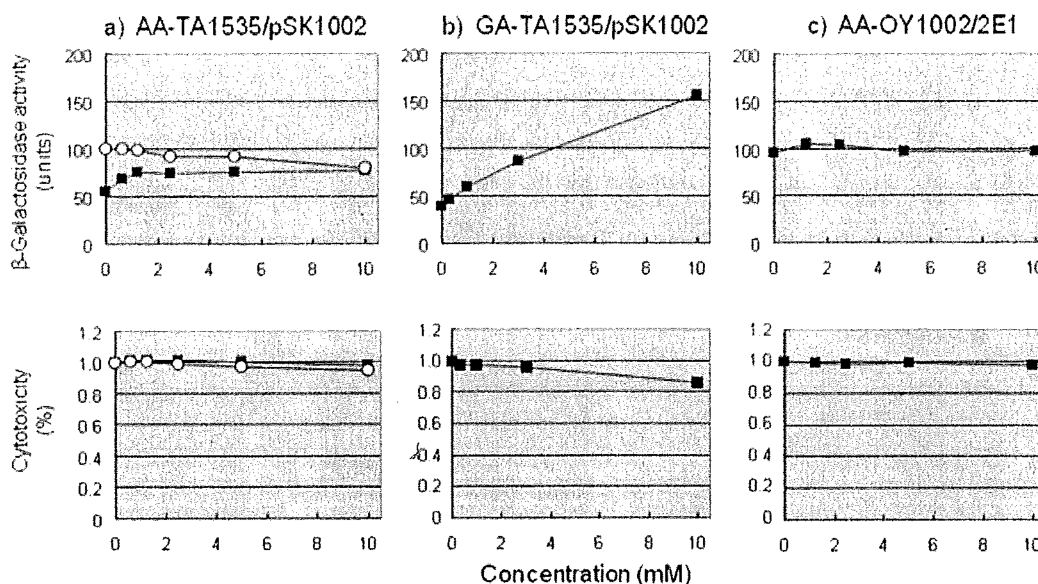

Fig. 2. Induction of *umuC* gene expression and cytotoxic response by AA (a, c) or GA (b) in *S. typhimurium* tester strains TA1535/pSK1002 (a, b) and OY1002/2E1 (c). The *umu* tests were conducted without (■) or with rat S9 (○). β-Galactosidase activity (units) was determined as described in Materials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

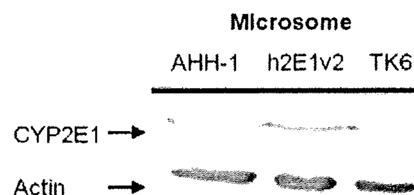

Fig. 3. Western blot analysis of CYP2E1 in AHH-1, h2E1v2, and TK6 cells. Equal amount of materials were loaded for each sample. CYP2E1 protein was stained with the anti-CYP2E1 antibody. Actin was used as a loading control.

adduct formation, and the in vitro metabolic activation system did not metabolize AA into GA.

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;

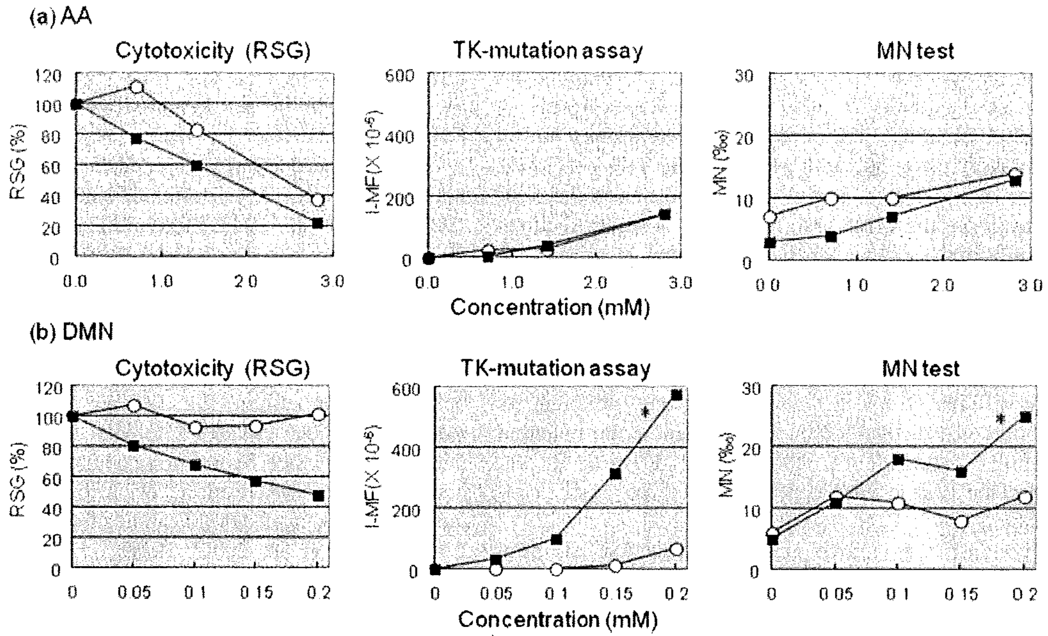

Fig. 4. Cytotoxic (relative suspension growth, RSG) and genotoxic (TK assay and MN test) responses of AHH-1 (○) or h2E1v2 (■) cells treated with AA or DMN for 4 hr. I-MF means induced mutation fraction, in which back ground mutation frequency is subtracted. *$P < 0.05$ (Omori method for TK-mutation assay,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

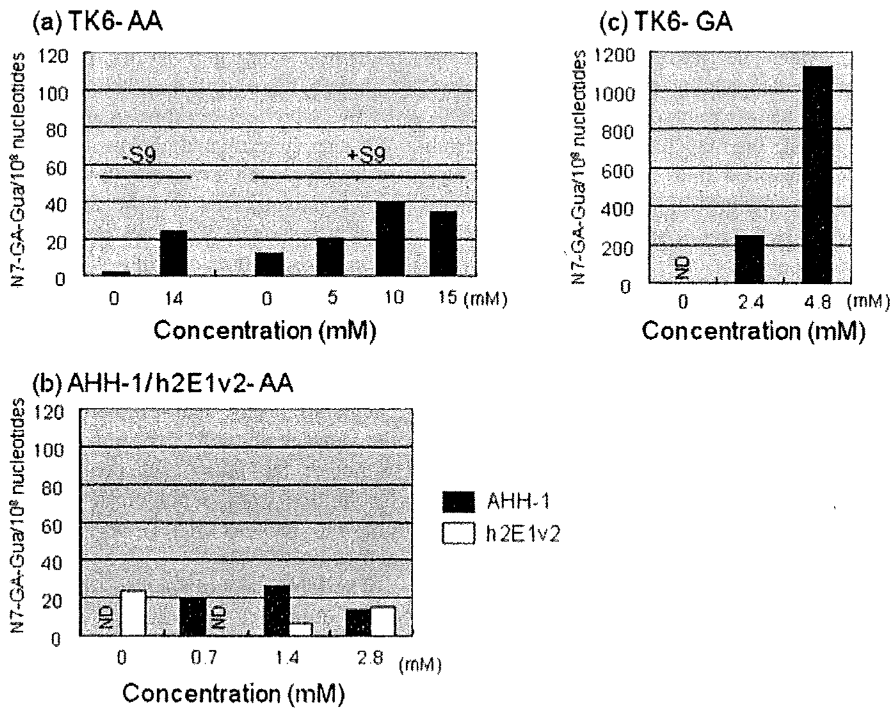

Fig. 5. Levels of N7-GA-Gua adduct in TK6 (a, c), AHH-1 (b), or h2E1v2 (b) cells treated with AA (a, b) or GA (c) for 4 hr at different concentrations. Data are expressed as the number of adducts in 10^8 nucleotides.

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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