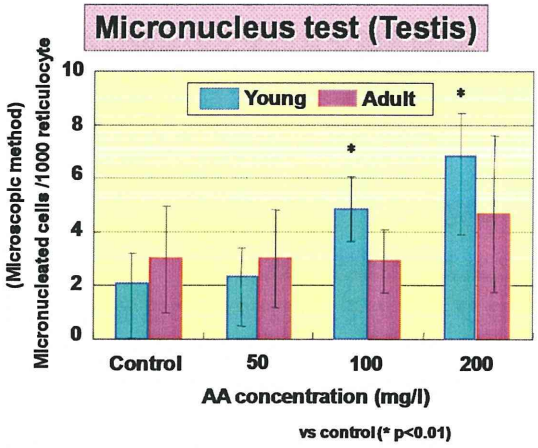

図 1

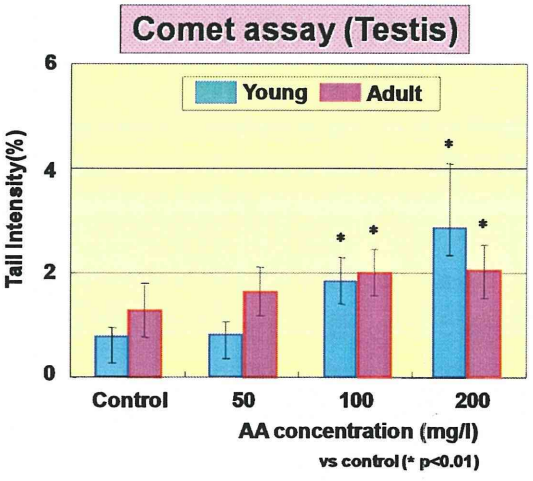

図 2

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 3）。

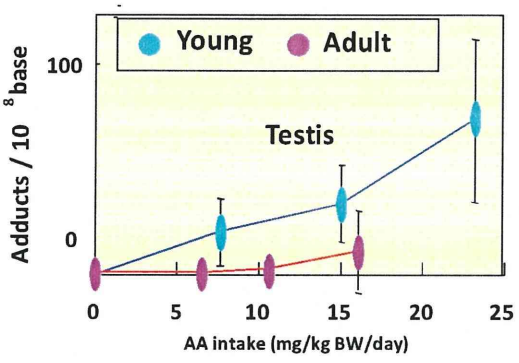

図 3

ii) ラット強制経口投与試験：

4、6、10 週齢 SD ラットに 25mg/kg、もしくは 50mg/kg の AA を強制経口投与し、最終投与 24 時間後に屠殺した。精巣の小核については 10 週齢のラットでは用量依存的な増加が観察されたが、4、6 週齢では小核の誘発は観察されなかった(図 4)。

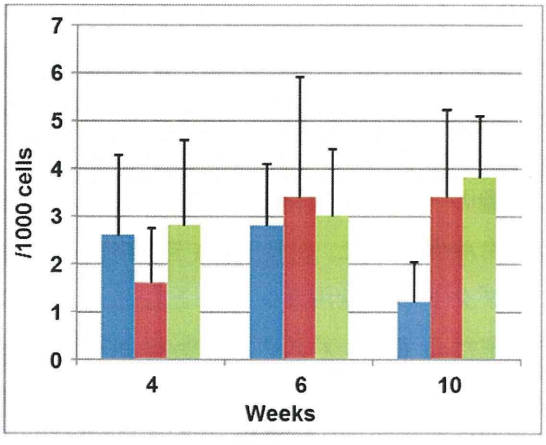

図 4

精巣のコメットは 4 週齢群では 50mg/kg 投与群で、6、10 週齢群では 25、50mg/kg 投与群で顕著な誘発が観察されたが、全ての群で用量依存性は明らかではなかった（図 5）。

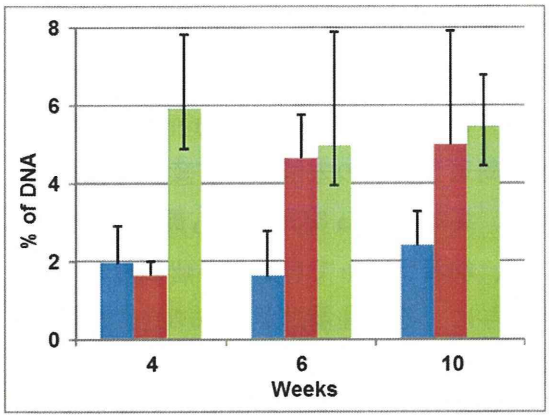

図 5

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。全ての週齢において DNA アダクトは用量依存的に増加した。DNA アダクト量は 4 週齢ラットが最も多く、次いで、6 週齢、10 週齢と蓄積量が減少した（図 6）。

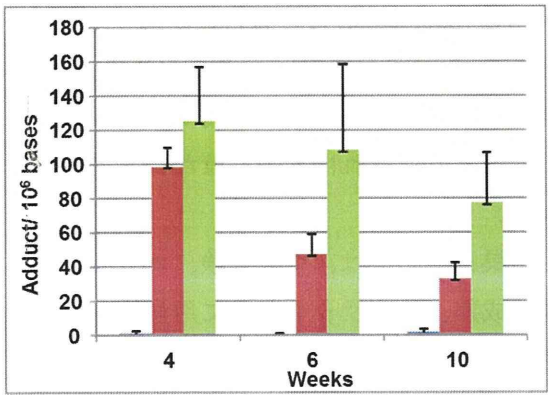

図 6

<u>iii) マウス飲水投与試験：</u>

3、10 週齢 gpt マウスに 100、200、400ppm の AA を自由飲水させ屠殺した。幼若マウスでの 400ppm は毒性強く、一部のマウスが死亡したため、その後の投与量を 300ppm へ減弱した。個体当たり、体重当たりの AA 摂取量は 200ppm までは 3、10 週齢では差が認められなかった。400ppm では 10 週齢マウスで AA 摂取量が高かったが、これは 3 週齢マウスでは強に毒性により飲水行動が困難だったためと考えられる。

赤血球の Pig-a 突然変異結果を図 1 に示す。

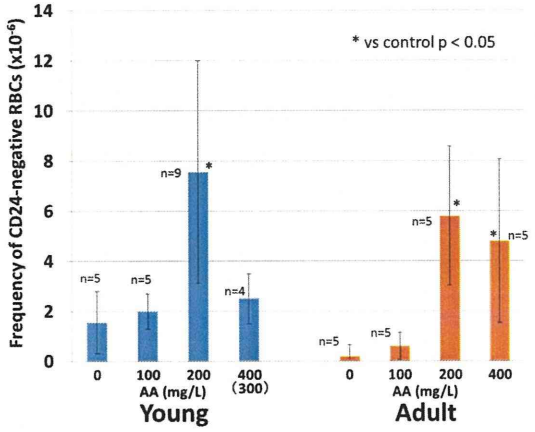

図 7

200ppm 以上で 3、10 週齢とも顕著な遺伝子突然変異の誘発が観察されたが、週齢による差は認められなかった。また、400ppm による突然変異頻度の抑制は細胞毒性に起因するものと考えられる。

精巣での gpt 突然変異の結果を図 8 に示す。

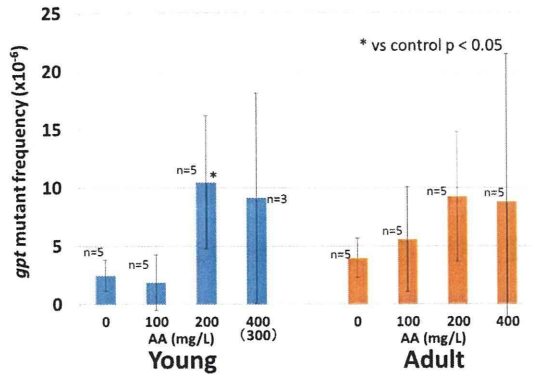

図 8

200ppm 以上で 3、10 週齢とも遺伝子突然変異の誘発が観察されたが、10 週齢では統計

的有意差が無かった。また、週齢による差も認められなかった。

精巣でのN7-GA-Guaアダクト量を図9に示す。

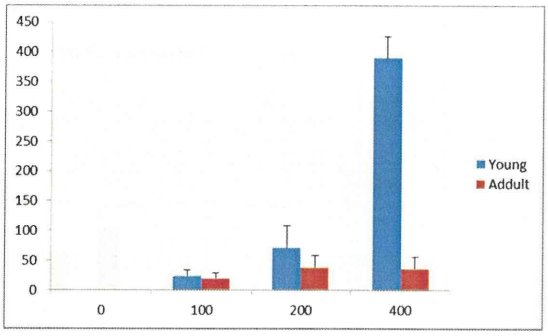

図9

DNAアダクト量は3、10週齢とも用量依存的に増加した。200ppm以上で週齢差が観察され、400ppm以上では3週齢の方が10週齢と比較して10倍程度のN7-GA-Guaの蓄積が観察された。

D．考　察

i) ラット飲水投与試験：

これまでAAのin vivoでの飲水投与による遺伝毒性研究に関しては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)。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、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、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。

ii) ラット強制経口投与試験：

飲水投与試験の結果を検証するため、4、6、10 週齢の SD ラットに 25、もしくは 50mg/kg の AA を 1 週間強制経口投与し、精巣での遺伝毒性（コメット試験、小核試験）と DNA アダクト量を検討した。飲水投与に用いた最高濃度である200ppmではAAの一日摂取量は 11 週齢ラットで 18mg/kg/day、3 週齢ラットで 25mg/kg/day であったことから、今回の我々の強制経口投与量ではその倍以上の AA を 1 週間摂取したことになる。

小核試験では AA による小核の誘発、また週齢差は顕著ではなかった。コメットは全ての週齢で、AAによる誘発が観察された。飲水投与の実験でも、小核の誘発は明らかでなく、コメットの誘発は用量依存的に誘発したことが観察された。精巣の遺伝毒性の評価にはコメット試験、遺伝突然変異試験が有効であるのかもしれない。しかしながら、AAは本来、精巣で強い染色体異常が観察されることが報告されている。効率的な精巣での遺伝毒性の検出にはさらなる検討が必要と考えられる。

精巣での N7-G-GA は全ての週齢のラットで用量依存的に増加し、特に 4 週齢でその蓄積量は高かった。また、6 週齢と 10 週齢の差は顕著ではなかった。4 週齢での 25mg/kg と 50mg/kg 投与での差は明らかでなく、高濃度ではアダクト生成が飽和しているものと考えられる。

幼若ラットでのアダクト生成の増加のメカニズムに関しては明らかではないが、最近 Takahashi らが精巣中のグルタチオントランスフェラーゼ（GST）の活性が、幼若ラットで有意に低いことを報告している。AAやGAはGSTによって縫合反応を受け、解毒されると考えられている。この解毒反応の低下が、幼若ラットの精巣でのアダクト量の増加を説明できるかもしれない。

iii) マウス飲水投与試験：

ラットで観察された、幼若動物の精巣での AA 遺伝毒性感受性をマウスを用いて検証を行った。3週、および10週齢の gpt delta マウス（雄）に 0、100、200、400ppm の AA を 28 日間自由飲水経口摂取させ、赤血球の Pig-a 遺伝子突然変異と、精巣の gpt 遺伝子突然変異を検討した。また、精巣ではAAのDNAアダクト(N7-GA-Gua)量も解析した。

200ppm 以上で Pig-a（赤血球）、gpt（精巣）突然変異とも有意に増加したが、週齢間で差は見られなかったが、DNA アダクト生成量（精巣）は、用量依存的に増加し、また幼若マウスでは蓄積量が顕著に高かった。体重当たりの AA 摂取量に週齢間で差は見られなかったことから、幼若期の精巣での AA の高蓄積性はライフステージに依存した AA の代謝に関連するものと考えられた。

DNA アダクト量が成熟、幼若動物で顕著である一方、他の遺伝毒性マーカの差は顕著ではなかった。この原因は不明であるが、今回の実験では幼若マウスの方が、同じ AA 摂取量であるにもかかわらず、より低濃度で毒性が発現したことから、幼若動物では AA の毒性に対して感受性が高く、細胞死に引き起こしやすいのかもしれない。N7-GA-Gua アダクト量はこれを反映しているため、毒性評価の有効なバイオマーカーとなり得るかもしれない。精巣での遺伝毒性の検出にはタイミングが重要と考えられ、経時的な遺伝毒性のモニタリングが必要である。

幼若期の精巣での AA の高蓄積性はライフステージに依存した AA の代謝に関連するものと考えられる。種間で共通して観察されたことから、ヒトに対する影響も考えられ、AA の発がんリスクの検討には、小児に対して特別の配慮が必要と考えられる。

E. 結　論

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する目的で、幼若、成熟ラット、マウスを用いて、飲水投与もしくは経口強制投与の動物実験を行い、AA の遺伝毒性反応性を比較検討した。概して、成熟、および幼若動物での遺伝毒性反応性は、骨髄や肝臓では顕著ではなかったが、精巣においては、幼若動物の方で高い傾向にあった。一方、DNA アダクト生成量は、幼若マウスの精巣で顕著であった。幼若期のラット、マウス精巣での AA の高蓄積性はライフステージに依存した AA の代謝に関連するものと考えられる。種間で共通して観察されたことから、ヒトに対する影響も考えられ、AA の発がんリスク評価には、小児に対しては特別の配慮が必要と考えられる。

F. 研究発表

1．論文発表

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Koyama N, Yasui M, Kimura A, Takami S, Suzuki T, Masumura K, Nohmi T, Masuda S, Kinae N, Matsuda T, Imai T, Honma M. Acrylamide genotoxicity in young versus adult gpt delta male rats. Mutagenesis. 26, 545-549 (2011)

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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
Cho YM, Imai T, et al.	Increased H-*ras* mutation frequency in mammary tumors of rats initiated with *N*-methyl-*N*-nitroso-urea (MNU) and treated with acrylamide.	J Toxicol Sci	34	407-412	2009
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Koyama N, Honma M, et al.	Genotoxicity of acrylamide in vitro: Acrylamide is not metabolically activated in standard in vitro systems.	Environ Mol Mutagen	52	12-19	2011
Takahashi M, Honma M, et al.	Life stage-related differences in susceptibility to acrylamide-induced neural and testicular toxicity.	Arch Toxicol	85	1109-1120	2011

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Vol.34, No.4, 407-412, 2009

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*]anthr acene (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 (cm^3) [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 (cm^3) [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 (cm^3) [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

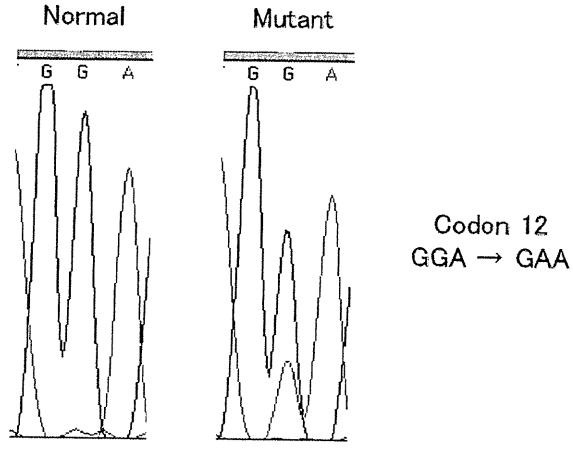

Fig. 1. Results of direct sequencing analysis of codon 12 of H-*ras* in MNU-induced mammary tumors. The mutant sample demonstrates an extra-signal for A in the second base indicating the presence of a mutation.

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

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Juvenile rats do not exhibit elevated sensitivity to acrylamide toxicity after oral administration for 12 weeks

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ABSTRACT: Acrylamide (AA), a neurotoxic, testicular toxic, genotoxic and carcinogenic chemical, has been reported to be formed in processed food, and sensitivity to AA intoxication in childhood is a concern. In the present study, to clarify the general toxicological profile of AA in juvenile rats, subchronic toxicity was evaluated in F344 rats administered AA in the drinking water at 0 (control), 10, 20 and 40 ppm, presented to the dams (three per group) immediately after the birth of their litters, through lactation (3 weeks), and directly to the offspring in their drinking water after weaning for a further 9 weeks (12 weeks total). Treatment with AA caused a decrease in body weights in 20 and 40 ppm F_1 females, compared with the controls. Average AA intake throughout the treatment period for the 10, 20 and 40 ppm groups after weaning was equivalent to 1.0, 2.1 and 4.4 mg kg^{-1} body weight per day, respectively, in males and 1.2, 2.5 and 4.9 mg kg^{-1} body weight per day, respectively, in females. No toxicologically significant organ weight changes were observed. AA-induced histopathological changes were limited to focal degeneration and necrosis of the seminiferous epithelium in the testes and desquamated epithelium in the ducts of epididymides, noted only in 40 ppm males. Taken together with previous reports, juvenile rats are not necessarily more susceptible to AA-induced toxicity as compared with young adults. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: acrylamide; juvenile rats; histopathology

INTRODUCTION

Acrylamide (AA), a vinyl monomer that improves the aqueous solubility, adhesion and cross-linking of polymers, is used as a mobility control agent for oil recovery, a flocculant for wastewater treatment and soil stabilization and a reagent for scientific research (IARC, 1994). There are many reports demonstrating various toxic effects of AA in experimental animals, including neurotoxicity (Burek *et al.*, 1980; LoPachin *et al.*, 2003; Tyl *et al.*, 2000a), testicular toxicity (Burek *et al.*, 1980; Yang *et al.*, 2005), reproductive toxicity (Tyl *et al.*, 2000a, b) and genotoxicity (Maniere *et al.*, 2005; Paulsson *et al.*, 2003; Yang *et al.*, 2005). In addition, AA increased the incidence of lung and skin tumors in mice and consistently induced mesotheliomas, thyroid follicular cell and mammary tumors in two carcinogenicity studies in rats (Bull *et al.*, 1984a, b; Friedman *et al.*, 1995; Johnson *et al.*, 1986). The International Agency for Research on Cancer (IARC) has determined that AA is probably carcinogenic to humans (group 2A; IARC, 1994).

There have been several peripheral neuropathy cases as a result of AA intoxication by potential occupational exposure in man (Garland and Patterson, 1967; IARC, 1994; Spencer and Schaumburg, 1974, 1975), but environmental exposure under natural conditions, except for cigarette smoking, was previously thought to be excluded and controllable in the general population (Bergmark, 1997; IARC, 1994; Spencer and Schaumburg, 1975). However, in 2002, new analytical data from Swedish scientists showed spontaneous AA formation in fried and baked foods at various concentrations, e.g. moderate levels of AA (5–50 μg kg^{-1}) were detectable in heated protein-rich foods and higher contents (150–4000 μg kg^{-1}) were detectable in carbohydrate-rich foods, while AA could not be measured in unheated control or boiled foods (<5 μg kg^{-1}) (Tareke *et al.*, 2002). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that the average AA intake for the general population and high consumers was approximately 1 and 4 μg kg^{-1} body weight per day, respectively, with margin of exposure (MOE) values of 200 and 50 for morphological changes, such as demyelination and/or degeneration of axons in nerves in rats, 310 and 78 for induction of mammary tumors in rats and 180 and 45 for induction of Harderian gland tumors in mice. These MOE values are relatively low compared with other genotoxic contaminants in foods (JECFA, 2010). Furthermore, human exposure levels to AA from cooked foods during childhood are estimated to be higher than in adults

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(Dybing *et al.*, 2005; Hartmann *et al.*, 2008; JECFA, 2005; Konings *et al.*, 2003). Neurotoxicity, genotoxicity and testicular toxicity have already been demonstrated in rats administered AA during young adult stages and a rat two-generation study (Tyl *et al.*, 2000a), as described above, but experimental data showing sensitivity or target organ/tissue differences in rats administered AA in fetal and/or infant stages are limited (Friedman *et al.*, 1999; Garey *et al.*, 2005; Takahashi *et al.*, 2008, 2009; Wise *et al.*, 1995).

In this study, to clarify the general toxicological profile of AA in the juvenile stage, male and female F344 rats were administered AA in their drinking water after birth for 12 weeks. The results were evaluated by referring to previously reported data in AA toxicological studies in young adult and adult rats, and suggested that juvenile rats are not more susceptible to AA-induced toxicity than young adults.

MATERIALS AND METHODS

Chemical and Antibody

AA (CAS no. 79-06-1, purity ≥ 99%) was purchased from Sigma-Aldrich (St Louis, MO, USA), and mouse monoclonal antibodies to proliferating cell nuclear antigen (PCNA; clone PC10) were purchased from DakoCytomation (Glostrup, Denmark).

Animal Treatments

For experiments 1 and 2, 12 and 10 specific pathogen-free pregnant F344 rats (F344/DuCrlCrlj), respectively, all synchronously mated at 10 weeks of age, were purchased from Charles River Laboratories Japan (Kanagawa, Japan). The animals were individually housed in clear polycarbonate cages with sterilized white wood chips (Sankyo Laboratory Service, Tokyo, Japan) for bedding in a standard air-conditioned room (24 ± 1 °C, 55 ± 5% relative humidity, 12 h light/dark cycle) and were given basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and tap water *ad libitum* until parturition.

Experimental Protocol

Experiment 1

Three dams in four groups each were given free access to AA-containing drinking water at concentrations of 0 (control), 10, 20 and 40 ppm for the 3 weeks of lactation after parturition. Three days after birth, the F_1 litters were standardized by randomly selecting four males and four females per litter (in principle), to maximize the uniformity of growth rates of the offspring. The mean litter size at birth in the 0, 10, 20 and 40 ppm groups was 8.3, 8.6, 8.0 and 8.6, respectively. The sex ratio in the 0, 10, 20 and 40 ppm groups was 28, 54, 54 and 50%, respectively, for male, and 72, 46, 46 and 50%, respectively, for female. The number of offspring in the 0, 10, 20 and 40 ppm groups was 7, 12, 13 and 12, respectively, for male, and 17, 12, 11 and 12, respectively, for female. After weaning at 3 weeks of age, the dams were euthanized by exsanguination from the thoracic aorta under deep ether anesthesia, and the offspring were maintained on the same concentrations of AA in their drinking water as their dams for a further 9 weeks. The maximum dose level of AA for the present experiment was equivalent to those used in previous reports demonstrating that young adult rats receiving 5.0 mg kg^{-1} body weight per day AA for 13 weeks revealed slight peripheral nerve degeneration but not neurotoxicological symptoms (Burek *et al.*, 1980). Drinking water containing AA was replaced once a week.

During the experimental period, a check for clinical signs and mortality was performed at least once daily. Individual body weights and food and water consumption per cage were measured once a week. At the end of the experiment, all surviving animals were euthanized by exsanguination from the thoracic aorta under deep ether anesthesia, and subjected to a complete necropsy with the major organs and tissues examined macroscopically. Brain, thymus, lungs, heart, spleen, liver, adrenals, kidneys and testes were excised and weighed. In addition to these organs, the nasal cavity, trachea, aorta, pituitary, thyroids, parathyroids, salivary glands, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, urinary bladder, epididymides, prostate, seminal vesicles, bulbourethral glands, ovaries, uterus, vagina, mammary glands, skin, cervical and mesenteric lymph nodes, sternum and femur including bone marrow, trigeminal nerve, sciatic nerve (left), spinal cord (cervical, thoracic and lumber cord), eyes, Harderian glands, thigh muscle and gross abnormalities were excised. All these organs and tissues were fixed in 10% buffered formalin, except for the testes, which were fixed in Bouin's solution. The thyroids were weighed after fixation. All fixed samples were trimmed for embedding in paraffin and routinely processed to hematoxylin and eosin (HE) stained sections. For histopathological assessment of axons in the peripheral nerves, the sciatic nerves (right) were exposed and subjected to *in situ* fixation by immersion in ice-cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 min. Dissected sciatic nerves were further fixed with the same fresh fixative overnight, postfixed in 1% osmium tetroxide in the same buffer for 2 h at 4 °C, and embedded in epoxy resin (TAAB Laboratories Equipment, Berkshire, UK). Semithin sections, 1 μm in thickness, were stained with toluidine blue. Histopathological examination was performed on all organs and tissues of animals in the control and high-dose groups of both sexes. If a chemical treatment-related change appeared at the high dose, the relevant organ/tissue(s) from the lower dose groups was then also examined. In addition, thyroid sections of all groups, including the controls, were immunohistochemically stained for PCNA using anti-PCNA mouse monoclonal antibodies diluted at 1:100 and a streptavidin–biotin–peroxidase complex kit (StreptABComplex/HRP, DakoCytomation), with the chromogen 3,3′-diaminobenzidine followed by counterstaining with hematoxylin. The numbers of cells positive for PCNA were counted to determine percentage values with at least 1000 follicular epithelial cells in each thyroid section.

Experiment 2

To confirm whether the apparent increase in the incidence and severity of myocarditis in the high-dose males and decrease in the incidence of calcification in renal tubular epithelium in the high-dose females in experiment 1 were truly AA-specific reactions, the second experiment was conducted. Five dams each in two groups were given free access to AA-containing drinking water at concentrations of 0 (control) or 40 ppm (high dose) during lactation (3 weeks) and then, once weaned, the offspring were maintained on the same concentrations of AA in their drinking water as their dams for 9 weeks (12 week total). The mean litter size at birth in the 0 and 40 ppm groups was 9.6 and 9.0, respectively. The sex ratio in the 0 and 40 ppm groups was 48 and 47% male,

respectively. Twenty offspring of both sexes were obtained in the 0 and 40 ppm groups each. At the end of the experiment, all animals were fasted overnight and euthanized by exsanguination with blood sample collection from the abdominal aorta under deep ether anesthesia for serum biochemistry assays performed at SRL (Tokyo, Japan) for aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), calcium (Ca) and inorganic phosphorus (IP) as markers of cardiac and/or renal toxicities.

All animals were subjected to complete necropsy, and the major organs/tissues were all examined macroscopically. The heart and kidneys (females only) were excised and weighed. These organs were fixed in 10% buffered formalin, and fixed samples were trimmed for embedding in paraffin and routinely processed to HE-stained sections for histopathological examination.

The experimental protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Statistics

The sample unit throughout the experiment, including the lactation period, was an individual pup/animal not a litter, since the principal purpose of the present study is to clarify the general toxicological profile in juvenile rats, and the treatment with AA was not performed on the F_0 dam during the gestation. Variance in values for body weights and serum biochemistry, organ weights and PCNA-positivity of thyroids was checked for homogeneity using Bartlett's procedure. When the data were homogeneous, one-way analysis of variance (ANOVA) was used. In the heterogeneous cases, Kruskal–Wallis's test was applied. When statistically significant differences were indicated, Dunnett's multiple test was employed for comparison between control and treated groups. With histopathological changes, incidences were compared using Fisher's exact probability test and severity data were analyzed with Mann–Whitney's *U*-test.

RESULTS

Experiment 1

In-life parameters

One male animal in the 10 ppm group died on day 83. The cause of death was cachexia due to a leiomyosarcoma of the stomach, which could have been a spontaneous lesion. Therefore this case was excluded from the evaluation of organ weights and histopathology. No other obvious clinical abnormalities, including neurotoxicological symptoms, were found in any of the groups throughout the experimental period. A slight but statistically significant reduction of body weight was noted intermittently (3–9 weeks of experiment) in 20 and 40 ppm females as compared with the controls (Fig. 1). Food and water consumption was similar among the groups throughout the experimental period. The average daily intake of AA in the 10, 20 and 40 ppm groups was 1.0, 2.1 and 4.4 mg kg^{-1} body weight, respectively, for males, and 1.2, 2.5 and 4.9 mg kg^{-1} body weight for females, showing a good correlation with the expected doses.

Organ weights

Data for final body and organ weights are summarized in Tables 1 and 2. There were no significant differences in final body weight among the groups in both sexes. In males, no significant differences were observed in any organs among the groups. In females, a decrease was observed in absolute thyroid weights in the 10 ppm group, heart weights in the 20 and 40 ppm groups and brain weights in the 40 ppm group. In addition, the relative thyroid weights in the 10 ppm group were decreased, and the relative thyroid and spleen weights in the 40 ppm group were increased.

Necropsy/histopathology

There were no obvious macroscopic findings in either sex in any of the groups. The results of histopathological examination are summarized in Table 3. Increased incidences of focal degeneration and necrosis of the seminiferous epithelium in the testes and desquamated epithelial cells in the epididymal tubules were observed in 40 ppm males (Figs 2 and 3), along with a statistically significant increase in the incidence and severity of myocarditis in the heart (Fig. 4). In females, the incidence of calcification in the renal tubular epithelium was reduced with/without statistical significance in the treatment groups. In the brain, spinal cord and trigeminal and sciatic nerves, no treatment-related histopathological changes were observed in either sex in any of the groups. PCNA-labeling indices of thyroid follicular cells in the 0, 10, 20 and 40 ppm groups were 0.95 ± 0.95 (mean ± SD), 0.82 ± 0.50, 0.89 ± 0.42 and 0.51 ± 0.38%, respectively, in males and 0.16 ± 0.19, 0.29 ± 0.11, 0.33 ± 0.21 and 0.22 ± 0.10%, respectively, in females, showing statistical significance ($P < 0.05$) in the 20 ppm female group but lacking dose dependence.

Experiment 2

In-life parameters

One control male was excluded from the evaluation because of severe growth depression due to malocclusion of the incisors. No other obvious clinical abnormalities including neurotoxicological symptoms were observed throughout the experimental period. Lowered body weights were noted in both sexes of the 40 ppm group as compared with the controls (Fig. 5). Food and water consumption was similar among the groups throughout the experimental period. The average daily intake of AA in males and females was 5.0 and 5.5 mg kg^{-1} body weight, respectively, showing a slight increase as compared with the expected dose, most likely because of a slight body weight reduction in both sexes during this period.

Serum biochemistry

Serum biochemistry data are summarized in Table 4. There was no treatment-dependent variation in either sex.

Organ weights

Data for final body and organ weights are summarized in Table 5. Significant reduction of final body weight was observed in the 40 ppm group in both sexes. The absolute heart weights in males and the absolute heart and kidney weights in females were decreased in the 40 ppm group.

Necropsy/histopathology

There were no obvious macroscopic findings in either sex in either group. Histopathological findings are summarized in Table 6. Myocarditis in the heart in both sexes and calcification in the renal tubular epithelium in females were observed in both control and 40 ppm groups, but the incidences and severity of these lesions were comparable between the two groups.

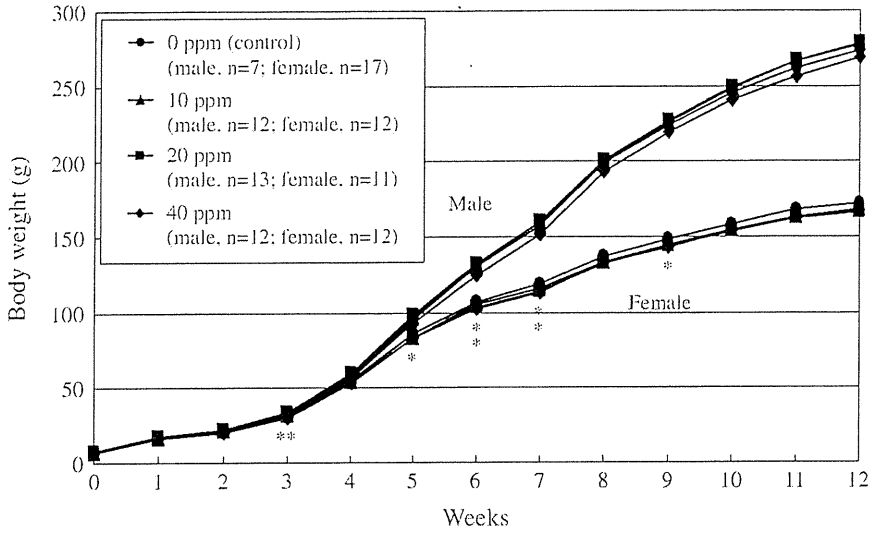

Figure 1. Experiment 1 – body weight curves for rats given drinking water containing acrylamide for 12 weeks. *, **Significantly different from the controls at $P < 0.05$ and 0.01, respectively.

Table 1. Experiment 1–Final body and organ weights of male rats administered water containing acrylamide for 12 weeks

	Dose level (ppm)			
	0 (control)	10	20	40
	No. of animals			
Items	7	11	13	12
Final body weights (g)	274.2 ± 10.0	276.3 ± 12.2	278.9 ± 10.7	268.4 ± 13.5
Absolute organ weights (g)				
Brain	1.91 ± 0.03	1.92 ± 0.01	1.93 ± 0.03	1.90 ± 0.04
Thyroids	0.016 ± 0.004	0.015 ± 0.002	0.014 ± 0.002	0.014 ± 0.004
Thymus	0.28 ± 0.02	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.01
Lungs	0.96 ± 0.07	0.96 ± 0.08	1.01 ± 0.09	0.94 ± 0.07
Heart	0.80 ± 0.05	0.80 ± 0.04	0.83 ± 0.04	0.80 ± 0.04
Spleen	0.60 ± 0.20	0.60 ± 0.03	0.61 ± 0.03	0.60 ± 0.04
Liver	10.34 ± 0.40	10.20 ± 0.78	10.55 ± 0.94	10.26 ± 0.83
Adrenals	0.033 ± 0.005	0.032 ± 0.005	0.032 ± 0.004	0.031 ± 0.004
Kidneys	1.79 ± 0.06	1.84 ± 0.13	1.86 ± 0.12	1.82 ± 0.12
Testes	2.90 ± 0.07	2.87 ± 0.11	2.95 ± 0.06	2.83 ± 0.10
Relative organ weights (g 100 g^{-1} body weight)				
Brain	0.70 ± 0.03	0.70 ± 0.03	0.69 ± 0.02	0.71 ± 0.03
Thyroids	0.006 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001
Thymus	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
Lungs	0.35 ± 0.02	0.35 ± 0.03	0.36 ± 0.02	0.35 ± 0.02
Heart	0.29 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.30 ± 0.01
Spleen	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.00	0.23 ± 0.01
Liver	3.77 ± 0.16	3.68 ± 0.19	3.78 ± 0.28	3.82 ± 0.21
Adrenals	0.012 ± 0.002	0.012 ± 0.002	0.012 ± 0.001	0.011 ± 0.001
Kidneys	0.65 ± 0.02	0.66 ± 0.02	0.67 ± 0.03	0.68 ± 0.03
Testes	1.06 ± 0.04	1.04 ± 0.05	1.06 ± 0.03	1.06 ± 0.05

Data are mean ± SD values.

DISCUSSION

In the present study, toxicological findings related to AA administration in juvenile rats were limited to reduced body weight gain and histopathological abnormalities in the testes and epididymides. Reduction in body weight was observed intermittently in females given 20 and 40 ppm (2.5 and 4.9 mg kg^{-1} body weight per day, respectively) in experiment 1 (3–9 weeks of experiment) and males and females receiving 40 ppm (5.0 and 5.5 mg kg^{-1} body weight per day, respectively) in experiment 2 (1–12 weeks of experiment), without any change in their food consumption. These rats were treated with AA during the early

Table 2. Experiment 1–Final body and organ weights of female rats administered water containing acrylamide for 12 weeks

	Dose level (ppm)			
	0 (control)	10	20	40
	No. of animals			
Item	17	12	11	12
Final body weights (g)	170.7 ± 8.1	168.9 ± 6.2	164.7 ± 5.1	166.3 ± 6.6
Absolute organ weights (g)				
Brain	1.78 ± 0.04	1.76 ± 0.02	1.78 ± 0.02	1.73 ± 0.02**
Thyroids	0.012 ± 0.002	0.010 ± 0.001*	0.011 ± 0.001	0.014 ± 0.002
Thymus	0.23 ± 0.01	0.23 ± 0.02	0.22 ± 0.02	0.22 ± 0.01
Lungs	0.73 ± 0.04	0.71 ± 0.05	0.73 ± 0.04	0.72 ± 0.06
Heart	0.56 ± 0.03	0.54 ± 0.02	0.53 ± 0.02*	0.53 ± 0.03*
Spleen	0.42 ± 0.03	0.40 ± 0.02	0.41 ± 0.02	0.42 ± 0.01
Liver	5.04 ± 0.32	5.19 ± 0.40	5.11 ± 0.15	4.99 ± 0.36
Adrenals	0.040 ± 0.007	0.037 ± 0.007	0.042 ± 0.005	0.035 ± 0.005
Kidneys	1.16 ± 0.08	1.13 ± 0.06	1.11 ± 0.07	1.11 ± 0.05
Relative organ weights (g 100 g^{-1} body weight)				
Brain	1.05 ± 0.05	1.04 ± 0.04	1.08 ± 0.02	1.04 ± 0.04
Thyroids	0.007 ± 0.001	0.006 ± 0.000*	0.007 ± 0.001	0.009 ± 0.001*
Thymus	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Lungs	0.43 ± 0.02	0.42 ± 0.03	0.44 ± 0.02	0.43 ± 0.03
Heart	0.33 ± 0.02	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.01
Spleen	0.244 ± 0.012	0.238 ± 0.011	0.247 ± 0.011	0.254 ± 0.008*
Liver	2.96 ± 0.15	3.07 ± 0.19	3.10 ± 0.09	3.00 ± 0.14
Adrenals	0.024 ± 0.004	0.022 ± 0.004	0.025 ± 0.003	0.021 ± 0.003
Kidneys	0.68 ± 0.03	0.67 ± 0.04	0.67 ± 0.03	0.67 ± 0.03

Data are mean ± SD values.
*,** Significantly different from the control values at $P < 0.05$ and 0.01, respectively.

stage of life including lactational and prepubertal stages and were evaluated by referring to previously reported data on AA toxicology in young adult and adult rats. In a previous two-generation reproduction study, a decrease in body weight was observed in F_0 male and female F344 rats exposed to AA in their drinking water during their pre-breeding young adult and adult stages for 10 weeks at doses of 0.5, 2.0 and 5.0 mg kg^{-1} per day (Tyl *et al.*, 2000a). Therefore, reduction in body weight might be a typical toxicological change with AA, but juvenile rats cannot be considered more sensitive as compared with young adults. Because the lactational transfer of AA to Sprague–Dawley rat offspring upon maternal oral administration was limited (Takahashi *et al.*, 2009), maternal inanition resulting in a lactation and/or a nursing disorder, as reported previously (Friedman *et al.*, 1999), may be one of the causes of reduced body weight gain in this experiment.

Focal degeneration and necrosis of the seminiferous epithelium in the testes and desquamated epithelium in the ducts of epididymides were observed in juvenile males given 40 ppm (4.4 mg kg^{-1} body weight per day) of AA. Exposure to 5 mg kg^{-1} body weight per day of AA in young adult male F344 rats in their drinking water for 13 weeks, from 6 to 19 weeks of age, has been reported to induce seminiferous tubule atrophy (Burek *et al.*, 1980). Thus, it is possible that the testes in juvenile rats are more susceptible to AA-induced toxicity than those in young adult and adult rats but, if so, this is only marginal.

Previously, histopathological alterations such as demyelinated and/or degenerated axons in the sciatic nerves were reported in male and female rats in a 13-week study with administration of AA at a dose of 5.0 mg kg^{-1} body weight per day started in the young adult stage (Burek *et al.*, 1980) and a neurological symptom, leg splay, was detected in F_0 female rats given 5 mg kg^{-1} per day in a two-generation reproduction study (Tyl *et al.*, 2000a). However, in the present study, no histopathological changes in the sciatic nerves or neurological symptoms were detected in either sex of the 40 ppm group (males, 4.4 mg kg^{-1} body weight per day; females, 4.9 mg kg^{-1} body weight per day). Therefore, juvenile female rats might be more resistant to neurotoxicity caused by AA exposure than young adults.

The thyroid has been considered one of the targets of AA carcinogenicity based on long-term rat studies, in which AA was administered in drinking water at doses up to 2.0 or 3.0 mg kg^{-1} body weight per day (Friedman *et al.*, 1995; Johnson *et al.*, 1986). In the previous literature, controversial results were reported in young adult rats. In one study, AA was administered to 8-week-old male F344 rats at doses of 2.0 and 15 mg kg^{-1} body weight per day for 7, 14 and 28 days, and an increase in DNA synthesis analyzed by BrdU-labeling of thyroid follicular cells was observed (Lafferty *et al.*, 2004). Another study examined possible evidence for disruption of the hypothalamic–pituitary–thyroid axis. Male F344 rats (from 21 to 70 days of age) were treated with AA in drinking water at doses of 2.5, 10 and 50 mg kg^{-1} body weight per day for 14 days, followed by evaluation of serum levels of thyroid and pituitary hormones; target tissue expression of genes involved in hormone synthesis, release and receptors; neurotransmitters in the CNS that affect hormone homeostasis; and histopathological changes in target tissues. The results showed no evidence for systematic alteration of the hypothalamic–pituitary–thyroid axis; Ki-67 gene expression level, a marker of cell proliferation, was

Table 3. Experiment 1–Histopathological findings in rats administered water containing acrylamide for 12 weeks

Sex	Organ	Findings	Grade[a]	Dose level (ppm) 0	10	20	40
Male	No. of animals			7	11	13	12
	Heart						
		Myocarditis, focal and/or zonal	+	2 (29%)	4 (36%)	6 (46%)	5 (42%)*
			++	0	2 (18%)	1 (8%)	4 (33%)*
	Pituitary						
		Cyst, pars distalis	+	0	0	0	2 (17%)
		Cyst, pars intermedia	+	0	1 (9%)	0	1 (8%)
	Testis						
		Tubular atrophy, partial	+++	0	1 (9%)	0	0
		Degeneration and necrosis of seminiferous epithelium, focal	+	0	1 (9%)	0	8 (66%)**
	Epididymis						
		Desquamated seminiferous epithelial cells	+	0	0	0	8 (66%)**
	Prostate						
		Mononuclear cell infiltration, focal	+	1 (14%)	2 (18%)	3 (23%)	3 (25%)
			++	1 (14%)	0	1 (8%)	0
		Atrophy, focal	+	0	1 (9%)	1 (8%)	1 (8%)
Female	No. of animals			17	12	11	12
	Kidney						
		Calcification, renal tubular epithelium	+	16 (94%)	7 (58%)*	7 (64%)	6 (50%)*
	Heart						
		Myocarditis, focal	+	2 (12%)	2 (17%)	1 (9%)	4 (33%)
	Submandibular gland						
		Basophilic focus	+	1 (6%)	0	0	0
	Pituitary						
		Cyst, pars distalis	+	1 (6%)	1 (8%)	0	0

[a]+, Slight; ++, moderate; +++, severe.
*,**Significantly different from the control values at $P < 0.05$ and 0.01, respectively.

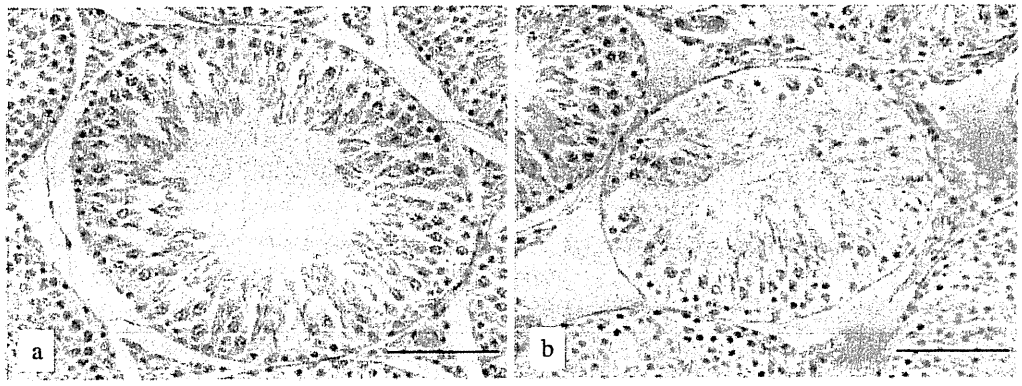

Figure 2. Experiment 1 – testis. (a) Control, a male. The seminiferous tubules of the testis are normal. The tubule in the central zone is in stage XIII. (b) The 40 ppm group, a male. Note the degeneration and necrosis of seminiferous tubules. The tubule in the central zone is in stages XII–XIII. Bar = 100 μm. HE staining.

not changed, and immunohistochemical Ki-67-positivity was decreased in the pituitary and thyroid by AA treatment (Bowyer *et al.*, 2008). In the present investigation, giving 4.4 mg kg^{-1} body weight per day of AA in male F344 rats for 12 weeks produced no obvious effects of AA on histopathology and a cell proliferative marker, PCNA-positivity, in thyroid follicular epithelial cells. Thus, juvenile rats may not be more susceptible to thyroid carcinogenesis after AA exposure.

An increase in the incidence and severity of myocarditis in 40 ppm males (9/12, 75%, vs 2/7, 29%, in the control) and a decrease in the incidence of calcification in the renal tubular epithelium in the treatment females (6/12, 50%, vs 16/17, 94%,

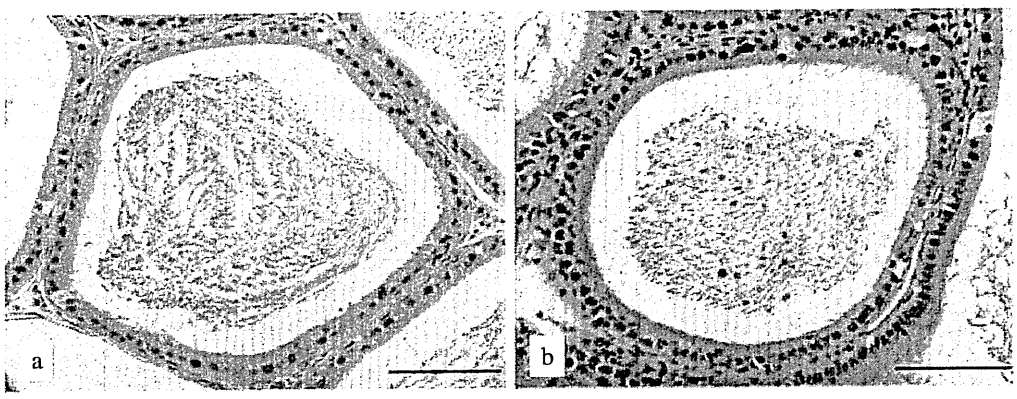

Figure 3. Experiment 1 – epididymis. (a) Control, a male. The epididymal ducts of the epididymis are normal. (b) The 40 ppm group, a male. Note desquamated epithelial cells in the epididymal ducts of the epididymis. Bar = 100 μm. HE staining.

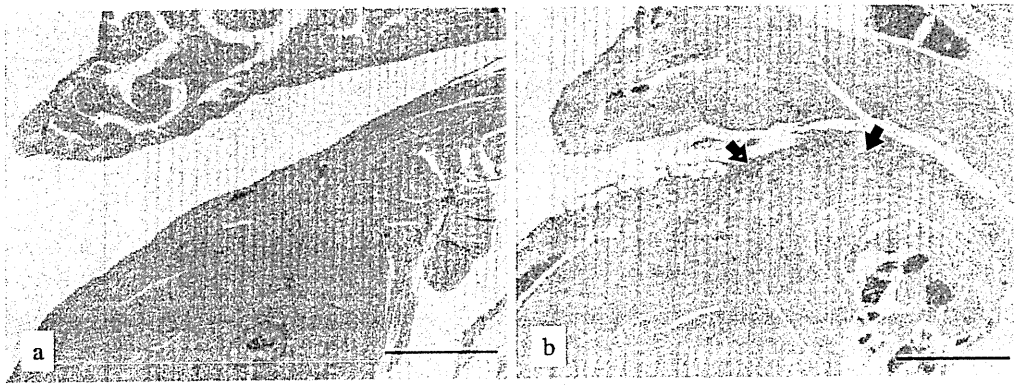

Figure 4. Experiment 1 – heart. (a) Control, a male. The side of the right ventricle of the heart is normal. (b) The 40 ppm group, a male. Note moderate infiltration of lymphocytes and macrophages with degeneration of myofibers (arrows) in the heart. Bar = 500 μm. HE staining.

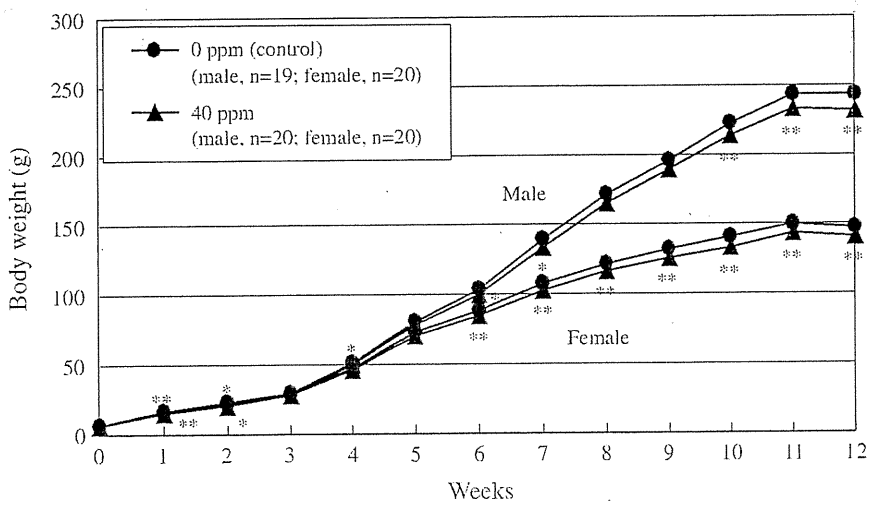

Figure 5. Experiment 2 – body weight curves for rats given drinking water containing acrylamide for 12 weeks. *, **Significantly different from the controls at $P < 0.05$, 0.01, respectively.

in the control) observed in experiment 1 were concluded to be incidental and not toxicologically significant for the following reasons. The incidence rates of myocarditis in control males and calcification in the kidneys in control females in this study may be low and high, respectively, compared with another study using male and female F344 control rats at our institute, in which the incidence of myocarditis in males was 40% and calcification in the kidneys in females was 10% (Takami *et al.*, 2008). Moreover, in a confirmatory study of experiment 2, there were no significant differences in the incidence and