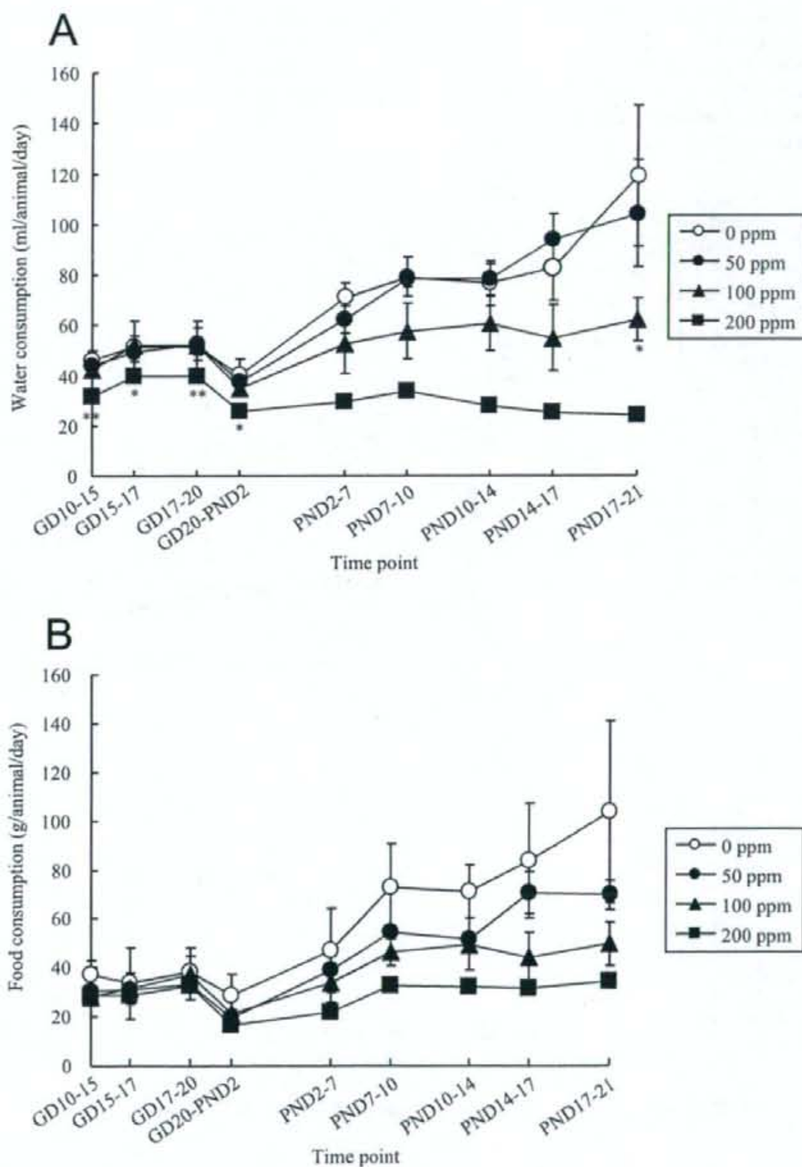

Fig. 1. Time course of change in water and food consumption by dams given ACR in the drinking water for the gestation and lactation periods. *A*. Water consumption. *B*. Food consumption. Data are mean ± SD. n=3 (0, 50 and 100 ppm group), n=3 or 2 (200 ppm group). *, **: $p<0.05$, $p<0.01$ vs. 0 ppm group. Abbreviation: GD, gestation day; PND, postnatal day.

females from 100 ppm at PNWs 4 and 5, and at 200 ppm thereafter (Fig. 6B).

At PNW 11, although some incidental findings were observed, no treatment-related histopathological changes were noted in the examined organs in either sex.

Morphometrically, no differences between the control and ACR-exposed groups were noted in parameters examined in the sciatic nerves and the cerebellum at PNW 11 (Table 4). The distribution of SYP-positive neuromuscular junctions was unchanged between the control and ACR-exposed groups at PNW 11.

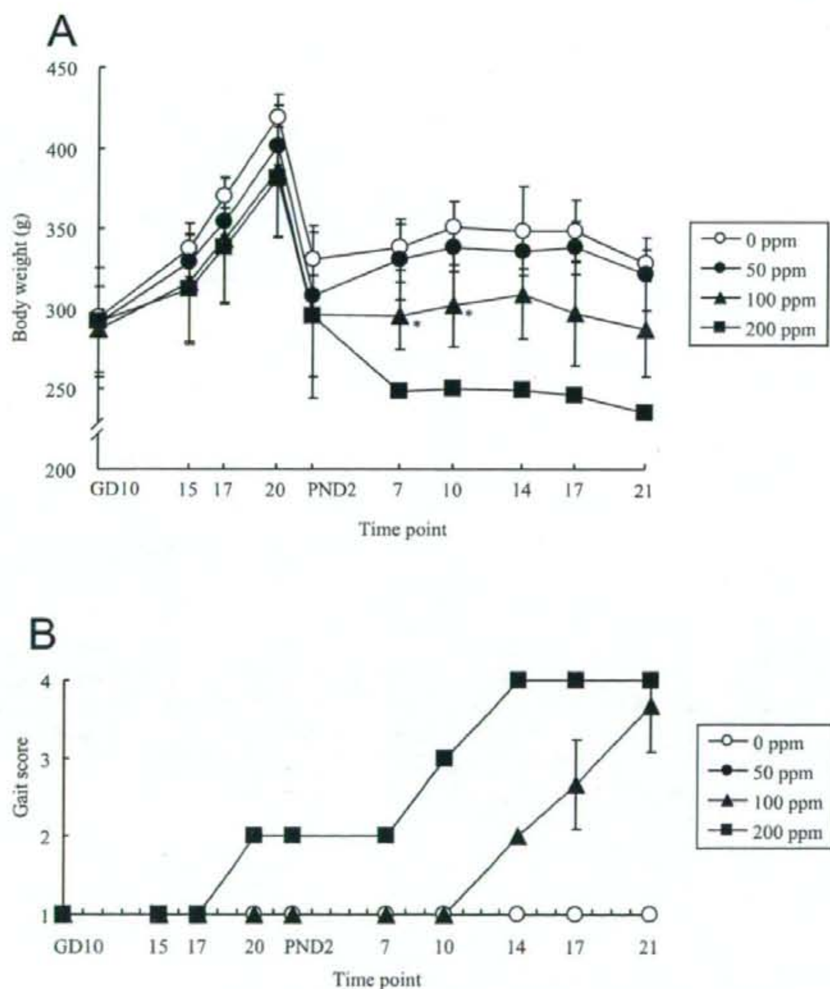

Fig. 2. Body weight changes and scores for gait abnormalities of dams given ACR in the drinking water for the gestation and lactation periods. *A*. Body weight. *B*. Scores for gait abnormalities. Data are mean ± SD. n=3 (0, 50 and 100 ppm group), n=3 or 2 (200 ppm group). *: $p<0.05$ vs. 0 ppm group. Abbreviation: GD, gestation day; PND, postnatal day.

DISCUSSION

In the present study, ACR-induced neurotoxicity in maternal animals was evident from 100 ppm on morphometric assessment of the sciatic nerves and cerebellar molecular layer, as previously reported (Lee *et al.*, 2005; Woo *et al.*, 2007). Dams given ACR at concentrations of ≥100 ppm exhibited depression of body weight and food and water consumption in a dose-dependent fashion. Maternal body weights at weaning were decreased by 12% and 27% relative to the control dams in the 100 and 200 ppm groups, respectively. In a previous study of the effects of feed restriction during the gestation and lactation periods in rats, body weights of dams were decreased by 1-5%, 10-20% and 17-32% in the 10%, 30% and 50% food restriction groups, respectively (Carney *et al.*, 2004), and the body weight curves were similar to those for our ACR-treated dams. Since reduced body weight gain concomitant with reduced food and water consumption was apparent here, particularly in the later stages of the experimental period, it can be considered that the advanced neurotoxicity due to ACR led to maternal malnutrition. ACR did not affect the gestation period, number of implantations, live birth ratio and male pup ratio, as in the report by Zenick *et al.* (Zenick *et al.*, 1986). Although inability to deliver was observed in one dam at 200 ppm, it was unclear whether

Table 1. Reproductive data.

	Acrylamide in the drinking water (ppm)			
	0	50	100	200
No. of animals	3	3	3	3
Gestational length (days)	22.0 ± 0.0[a]	22.0 ± 0.0	21.3 ± 0.6	21.7 ± 0.6
No. of implantations/dam	13.3 ± 0.6	13.7 ± 1.2	13.7 ± 2.1	13.3 ± 2.1
Live birth ratio (%)[b]	100	100	100	94
No. of live pups/litter[c]	12.7 ± 1.2	12.3 ± 2.5	11.3 ± 1.2	11.3 ± 3.8
No. of male pups[c]	8.7 ± 2.5	6.0 ± 4.6	5.0 ± 1.0	5.3 ± 2.3
No. of female pups[c]	4.0 ± 1.7	6.3 ± 3.2	6.3 ± 2.1	6.0 ± 3.6
Male pup ratio (%)	67.9 ± 15.6	46.7 ± 28.5	45.0 ± 13.6	49.1 ± 17.9
Male pup weight[c] (g)	8.27 ± 0.45	7.58 ± 0.66*	7.27 ± 0.65*	6.01 ± 0.39*
Female pup weight[c] (g)	7.97 ± 0.35	7.52 ± 0.43	6.67 ± 0.74*	5.59 ± 0.47*

[a]: Mean ± SD.
[b]: Live birth (%) = number of live pups delivered/total number of pups delivered ×100.
[c]: Measured at PND 2.
*: $p<0.01$ vs. 0 ppm group.

Table 2. Data for histopathology and morphometry of lesions developing in the nervous system of dams.

		Acrylamide in the drinking water (ppm)			
		0	50	100	200
No. of animals		3	3	3	3
Trigeminal nerve					
Central chromatolysis of ganglion cells (+/++)[a]		0	3 (3/0)	3 (0/3)	3 (0/3)
Sciatic nerve (distal portion)					
Density	(/100 μm²)	1.89 ± 0.31[b]	2.10 ± 0.11	1.96 ± 0.07	1.59[c]
Degenerated axons	(%)	1.04 ± 1.56	0.94 ± 0.20	2.43 ± 0.50*	5.78[c]
Myelinated axons, <3 μm in diameter (%)		7.82 ± 1.57	10.45 ± 2.80	13.02 ± 1.59*	14.55[c]
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.44 ± 0.11	0.75 ± 0.27	3.18 ± 0.45**	4.57[c]

[a]: Grade of change: +; mild, ++; moderate.
[b]: Mean ± SD.
[c]: One dam was killed due to inability to deliver.
*, **: $p<0.05$, $p<0.01$ vs. 0 ppm group.
Abbreviation: SYP, synaptophysin.

this was induced by ACR.

In the present study, pup body weights were significantly depressed at PND 2 from 50 ppm in males, and from 100 ppm in females, and decreased body weights persisted through PNW 11. A decrease of pup body weight has been previously reported in rats and mice with gestational exposure to ACR (Zenick *et al.*, 1986; Field *et al.*, 1990; Wise *et al.*, 1995), and such weight decrease has been proven to be a sensitive indicator of developmental ACR-toxicity, because it is apparent at dose levels lower than that producing maternal neurotoxicity (Wise *et al.*, 1995). This latter finding was also the case in the present study, suggesting that the decrease of pup body weight shortly after birth was caused by developmental effects of ACR administered during gestation. However, it has been reported that pup body weights were significantly decreased due to maternal malnutrition along with reduction of maternal body weight, food and water consumption in Wistar rats gavaged with ACR 25 mg/kg body weight/day throughout lactation (Friedman *et al.*, 1999). In addition, maternal food restriction during gestation and lactation suppresses pup body weight (Carney *et al.*, 2004).

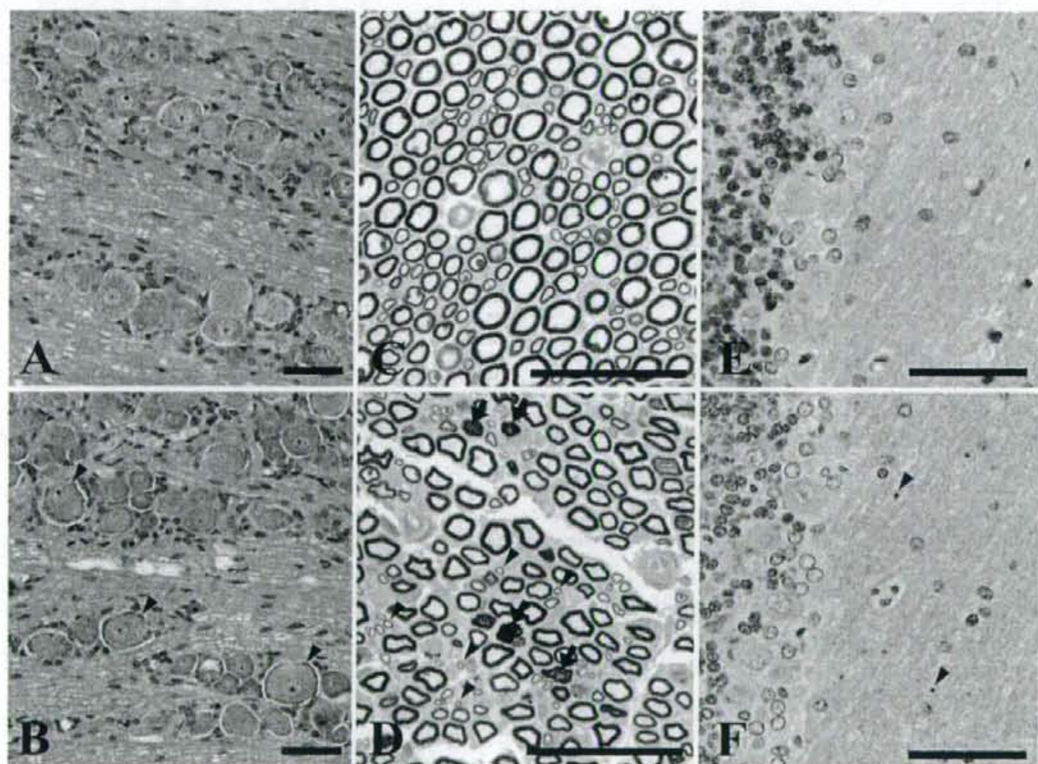

Fig. 3. Histopathology of the nervous system of dams given ACR at 0 (control) or 200 ppm in the drinking water for the gestation and lactation periods. (A) The trigeminal nerve of a dam at 0 ppm. (B) Central chromatolysis of ganglion cells (arrowheads) in the trigeminal nerve of a dam at 200 ppm. (C) The sciatic nerve of a dam at 0 ppm. (D) Increases of degenerated axons (arrows) and atrophic fibers (arrowheads) in the sciatic nerve of a dam at 200 ppm. (E) The cerebellar molecular layer of a dam at 0 ppm. (F) Increase of dot-like SYP-immunoreactive structures (arrowheads) in the cerebellar molecular layer at 200 ppm. A, B: hematoxylin and eosin. C, D: resin-embedded semithin sections stained with toluidine blue. E, F: immunohistochemical staining for SYP. Bar=50 μm.

Therefore, it is considered that the maternal malnutrition observed with ACR at ≥100 ppm could have greatly affected pup body weights in the present study. Although suppressed body weight persisted until PNW 11, differentiation of the effect of ACR toxicity from the influence of maternal malnutrition was difficult.

In the present study, neurotoxicity, represented by gait abnormalities in dams, was not found in offspring. Histopathologically, although findings suggesting developmental retardation and malnutrition, such as increase of retained external granular cells in the cerebellum, decrease of extramedullary hematopoiesis in the liver and spleen, and retardation of spermatogenesis, were found at weaning, it was difficult to separate direct effects of ACR on offspring from effects due to maternal toxicity. Increase of the axonal density and the proportion of small myelinated nerve fibers observed in the sciatic nerves at weaning might have been due to retarded growth accompanying maternal malnutrition rather than ACR-induced neurotoxicity, because the caliber of axons was reduced evenly in the ACR-treated groups. Moreover, degenerated axons in the sciatic nerves and aberrant dot-like SYP-immunoreactivity in the cerebellum, evident in dams, were not featured in the offspring at either weaning or PNW 11. Also, there were no other abnormalities indicating ACR toxicity in the nervous system at PNW 11. Therefore, the results suggest that ACR exposure through the maternal drinking water did not cause irreversible damage to the nervous system of offspring, at least under the present experimental conditions.

In a previous study, no signs of neurotoxicity were found in offspring after lactational ACR-exposure, and the authors considered that there was little possibility of ACR exposure to pups from the milk because of the maternal inanition (Friedman *et al*., 1999). Similarly, it is likely that ACR exposure through milk was limited in our study, since maternal malnutrition and neurotoxicity might have greatly influenced lactation. On the other hand, ACR has been reported to produce developmental neurotoxicity, including decreased motor activity and auditory startle responses, in SD rat offspring at maternal neurotoxic doses of 15 mg/kg body weight/day by oral gavage from GD 6 through PND 10 (Wise *et al*., 1995). Also, repeated oral doses of ACR up to 10 mg/kg body weight/day by gavage from GD 7 through delivery to maternal F344 rats and then to pups from PND 1 through PND 22 produced behavioral abnormalities and negative geotaxis performance at 10 mg/kg body weight/day with a linear trend in fall-time latencies on Rotarod performance on PND 21-22 (Garey *et al*., 2005). Therefore, pups may certainly exhibit behavioral abnormalities if exposed to ACR. In the present study, the mean daily intakes of ACR of dams during the

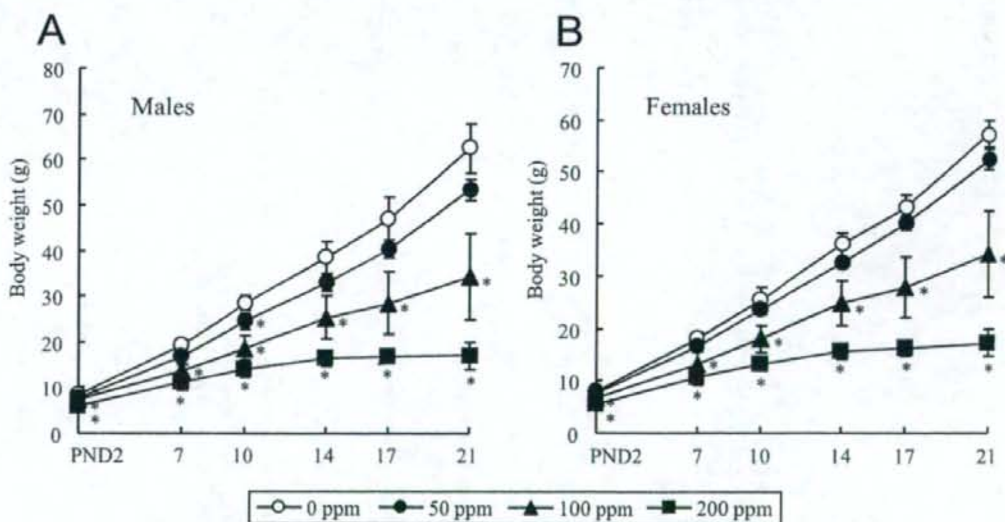

Fig. 4. Body weight changes of offspring before weaning. Data are mean ± SD. n=6 (0, 50 and 100 ppm group), n=4 (200 ppm group). *: p<0.01 vs. 0 ppm group. Abbreviation: PND, postnatal day.

Table 3. Data from histopathological analysis of F1 offspring at weaning.

	Males				Females			
	Acrylamide in the drinking water (ppm)				Acrylamide in the drinking water (ppm)			
Organ Findings	0	50	100	200	0	50	100	200
No. of animals	6	6	6	4	6	6	6	4
Cerebellum								
Increase of external granular cells (+/++/+++)[a]	6 (6/0/0)	6 (4/2/0)	6 (2/4/0)	4 (0/2/2) ††	6 (5/1/0)	6 (5/1/0)	6 (1/4/1)	4 (0/2/2) †
Liver								
Extramedullary hematopoiesis (±/+/++)[a]	6 (0/0/6)	6 (0/2/4)	6 (1/5/0) ††	2 (2/0/0) ††	6 (0/3/3)	6 (0/1/5)	5 (1/3/1)	4 (4/0/0) ††
Loss of cytoplasmic glycogen vacuoles, hepatocytes	0	0	0	3	0	0	0	2
Spleen								
Extramedullary hematopoiesis (±/+/++/+++)[a]	6 (0/0/0/6)	6 (0/0/2/4)	6 (0/3/3/0) ††	4 (4/0/0/0) ††	6 (0/0/5/1)	6 (0/0/6/0)	6 (1/3/2/0)	4 (3/1/0/0) ††
Testes								
Retardation of spermatogenesis (+/++/+++)[a]	0	0	6** (4/2/0) ††	4** (0/2/2) ††	–	–	–	–

[a]: Grade of change: ±: slight; +; mild, ++; moderate, +++: severe.
–: Not available.
**: $p<0.01$ vs. 0 ppm group (Fisher's exact test).
†, ††: $p<0.05$, $p<0.01$ vs. 0 ppm group (Mann-Whitney's *U*-test).

gestation and lactation periods were 16.7 ± 2.1 and 22.2 mg/kg body weight/day at 100, and 200 ppm, respectively, corresponding to the behaviorally neurotoxic dose to offspring in the above-mentioned study (Wise *et al.*, 1995). In man, fetal internal levels of ACR have been estimated to be at least equal to those achieved in mothers (Schettgen *et al.*, 2004). Therefore, the pups were likely to be exposed to ACR at a dose equivalent to that received by their dams during the gestation period at least. However, the duration of transplacental exposure in the present study from GD 10 through birth, within 2 weeks, might be too short to produce morphologically assessable lesions, since the dose used is known to induce neurotoxicity within 4 weeks in adult male SD rats (Lee *et al.*, 2005). Alternatively, the type and degree of neurotoxicity induced by developmental exposure of ACR may be different from the neurotoxic outcome apparent with progressive gait abnormalities observed in adult rats. Although the initial targets of ACR appear to be nerve terminals in both the central and peripheral nervous systems in adult animals (LoPachin *et al.*, 2003), these may be less sensitive to ACR-toxicity during development due to high neuronal plasticity.

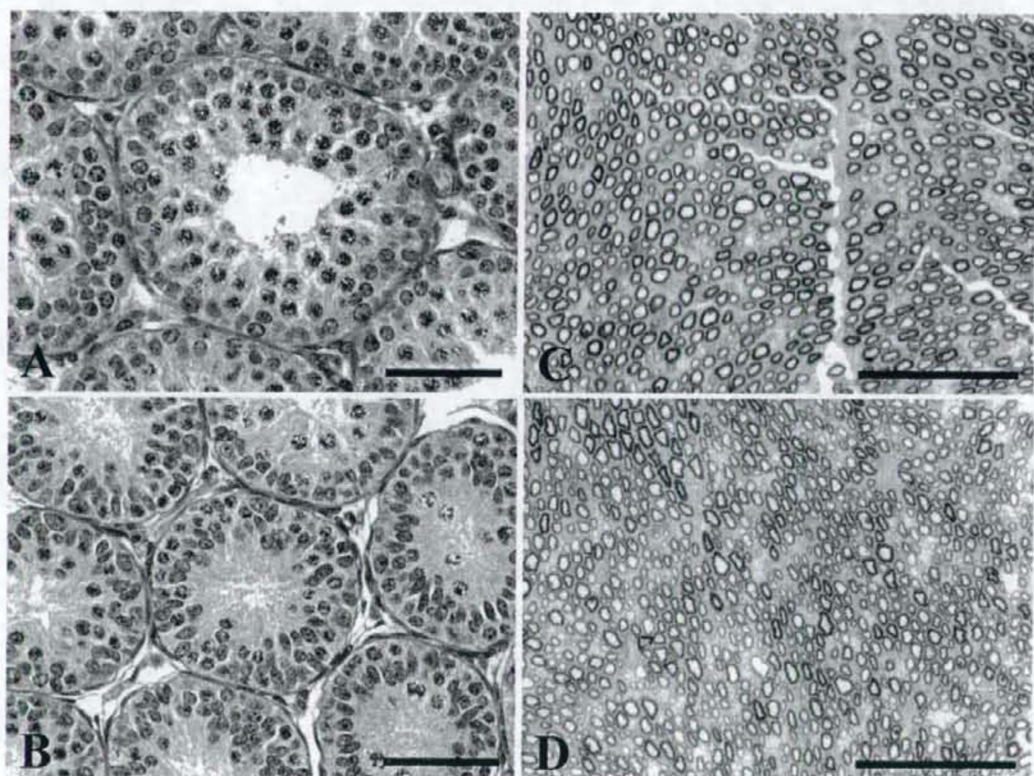

Fig. 5. Histopathology of the testis and sciatic nerve of pups at weaning exposed maternally to ACR at 0 (control) or 200 ppm for the gestation and lactation periods. (A) The seminiferous tubules showing 3–4 layers of spermatocytes in a male pup at 0 ppm. (B) The seminiferous tubules of a male pup at 200 ppm featured with 1–2 layers of gonocytes. Note reduced number of differentiated spermatocytes within the tubules as compared with the control animal. (C) The sciatic nerve of a male pup at 0 ppm. (D) The sciatic nerve of a male pup at 200 ppm. Note increase of nerve fiber density with small caliber as compared with the control animal. A, B: hematoxylin and eosin. C, D: resin-embedded semithin sections stained with toluidine blue. Bar=50 µm.

Regarding testicular toxicity, since it has been demonstrated that round spermatids are vulnerable to ACR (Sakamoto *et al.*, 1988), testes prior to spermatogenesis might be less sensitive. However, as with neurotoxicity, maternal malnutrition might have exerted a major effect, and further studies will be required to evaluate susceptibility to ACR-induced testicular toxicity in immature testes.

In conclusion, ACR administered in the maternal drinking water at concentrations of ≥100 ppm, while maternally neurotoxic, did not produce neurotoxicity or testicular toxicity in offspring under the experimental conditions applied here, despite decrease of pup weights at ≥50 ppm. Although there is a possibility that the target cells/cellular components of ACR in the adult case, such as the nerve

Table 4. Data for morphometry of lesions developing in the nervous systems of offspring at weaning and postnatal week 11.

		Acrylamide in the drinking water (ppm)			
		0	50	100	200
Weaning					
Males					
No. of animals		6	6	6	4
Sciatic nerve (distal portion)					
Density	(/100 μm²)	4.08 ± 0.34[a]	4.40 ± 0.22	4.47 ± 0.64	5.42 ± 0.37**
Degenerated axons	(%)	0.85 ± 0.17	0.67 ± 0.17	0.76 ± 0.20	0.72 ± 0.18
Myelinated axons, <3 μm in diameter	(%)	18.62 ± 2.32	19.11 ± 2.50	22.04 ± 5.08	25.51 ± 1.87*
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.29 ± 0.09	0.26 ± 0.11	0.32 ± 0.16	0.44 ± 0.09
Females					
No. of animals		6	6	6	4
Sciatic nerve (distal portion)					
Density	(/100 μm²)	4.26 ± 0.21	4.14 ± 0.48	4.73 ± 0.70	5.28 ± 1.27
Degenerated axons	(%)	0.82 ± 0.33	0.71 ± 0.18	0.81 ± 0.31	0.86 ± 0.38
Myelinated axons, <3 μm in diameter	(%)	20.93 ± 1.64	20.1 ± 2.55	22.37 ± 2.48	23.92 ± 8.21
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.55 ± 0.17	0.46 ± 0.17	0.55 ± 0.27	0.64 ± 0.33
Postnatal week 11					
Males					
No. of animals		6	6	6	4
Sciatic nerve (distal portion)					
Density	(/100 μm²)	1.87 ± 0.19	1.94 ± 0.19	1.83 ± 0.39	1.89 ± 0.16
Degenerated axons	(%)	1.06 ± 0.45	1.01 ± 0.55	0.93 ± 0.23	1.04 ± 0.19
Myelinated axons, <3 μm in diameter	(%)	9.50 ± 2.19	11.82 ± 1.75	11.38 ± 3.68	12.88 ± 1.03
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.58 ± 0.17	0.63 ± 0.28	0.53 ± 0.20	0.51 ± 0.12
Females					
No. of animals		6	6	6	3
Sciatic nerve (distal portion)					
Density	(/100 μm²)	2.22 ± 0.14	2.01 ± 0.27	2.08 ± 0.09	1.99 ± 0.11
Degenerated axons	(%)	1.11 ± 0.84	0.89 ± 0.40	1.08 ± 0.71	1.30 ± 0.23
Myelinated axons, <3 μm in diameter	(%)	11.47 ± 0.76	10.99 ± 2.01	11.41 ± 1.90	12.03 ± 2.39
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.74 ± 0.27	0.59 ± 0.36	0.61 ± 0.29	0.67 ± 0.38

[a]: Mean ± SD.
*, **: $p<0.05$, $p<0.01$ vs. 0 ppm group.
Abbreviation: SYP, synaptophysin.

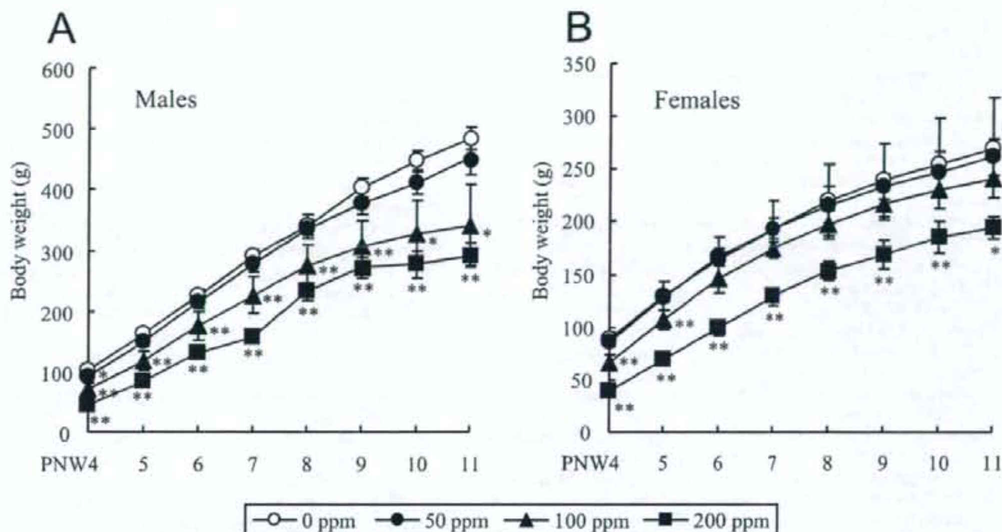

Fig. 6. Postweaning body weight changes of offspring. Data are mean ± SD. n=6 (0, 50 and 100 ppm group), n=4 or 3 (200 ppm group).*, **: $p<0.05$, $p< 0.01$ vs. 0 ppm group. Abbreviation: PNW, postnatal week.

terminals and seminiferous epithelium, might be less sensitive to ACR toxicity during development, limited exposure due to maternal toxicity during the lactational period may be the reason why pups did not show here any ACR-induced toxicity except for retarded body growth.

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Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells

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Abstract

The recent finding that acrylamide (AA), a potent carcinogen, is formed in foods during cooking raises human health concerns. In the present study, we investigated the genotoxicity of AA and its metabolite glycidamide (GA) in human lymphoblastoid TK6 cells examining three endpoints: DNA damage (comet assay), clastogenesis (micronucleus test) and gene mutation (thymidine kinase (*TK*) assay). In a 4 h treatment without metabolic activation, AA was mildly genotoxic in the micronucleus and *TK* assays at high concentrations (>10 mM), whereas GA was significantly and concentration-dependently genotoxic at all endpoints at $\geq$0.5 mM. Molecular analysis of the *TK* mutants revealed that AA predominantly induced loss of heterozygosity (LOH) mutation like spontaneous one while GA-induced primarily point mutations. These results indicate that the genotoxic characteristics of AA and GA were distinctly different: AA was clastogenic and GA was mutagenic. The cytotoxicity and genotoxicity of AA were not enhanced by metabolic activation (rat liver S9), implying that the rat liver S9 did not activate AA. We discuss the in vitro and in vivo genotoxicity of AA and GA.
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Keywords: Acrylamide; Gycidamide; Genotoxicity; *TK* mutation; Metabolic activation

1. Introduction

Acrylamide (AA) is a synthetic chemical that has been produced since the early 1950s. Because AA polymerizes easily to an adhesive gel, it has been widely used in industry for water flocculation, soil coagulation and grouts. Because it had been believed that humans are rarely exposed to AA under ordinary circumstances, concern was centered only on occupational exposure [1]. In 2000, however, Tareke et al. [2] reported that AA was unexpectedly discovered in cooking foods. It forms during frying and baking principally by a Maillard reaction between asparagine residues and glucose [3,4]. This finding raises concerns about the health risks of AA for the general population [5].

According to toxicological studies, AA is neurotoxic for animals and human [6,7], and the International

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Agency for Research on Cancer classifies it as 2A, a probable human carcinogen [1]. AA is also genotoxic in somatic and germinal cells in in vitro and in vivo [8]. In vivo examination [8] AA is metabolized to the epoxide derivative glycidamide (GA), presumably by cytochrome P4502E1 (CYP2E1) [9]. GA may be more toxic than AA because it reacts quickly with DNA and other biological macromolecules, and it is positive in most genotoxicity tests [8]. AA, on the other hand, is inactive in bacterial and some in vitro mammalian gene mutation assays, but it induces sister chromatid exchanges and chromosome aberrations in vitro and in vivo [8]. AA may have indirect genotoxic mechanisms, such as protein binding, spindle disturbance or hormonal imbalance, which could lead to tumors [10,11]. Thus, the genotoxic mechanism of AA is unclear.

In the present study, we used human lymphoblatoid TK6 cells to investigate the genotoxicity of AA and GA and its mechanisms. TK6 cells are widely used for the thymidine kinase (*TK*) gene mutation assay and can also be used in the in vitro micronucleus (MN) and comet (COM) assays. The *TK* gene mutation assay detects a wide range of genetic damage, including gene mutations, large-scale chromosomal deletions, recombination and aneuploidy [12], while other mammalian gene mutation assays, such as the *HPRT* and transgenic *LacZ* and *LacI* gene assays, detect only point mutations and small deletions [13]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumably relevant to carcinogenesis. Molecular analysis of the TK mutants induced by AA or GA can help elucidate their genotoxic mechanisms. In addition, because it uses a human cell line, the *TK* assay is appropriate for human hazard evaluation.

2. Materials and methods

2.1. Cell culture, chemicals and treatment

The TK6 human lymphoblastoid cell line has been described previously [14]. The cells were grown in RPMI1640 medium (Gibco-BRL, Life technology Inc., 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 maintained at 10^5 to 10^6 cells/ml at 37 °C in a 5% CO_2 atmosphere with 100% humidity.

AA (CAS # 79-06-1) and GA (CAS # 5694-00-8) were purchased from Wako Pure Chemical Co. (Tokyo). We dissolved them in phosphate-buffered saline just before use. *N*-di-*N*-butylnitrosamine (DBN) (CAS # 924-16-3) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and dissolved in DMSO for use. Post-mitochondrial supernatant fractions of liver homogenate (S9) were purchased from Kikkoman Co. Ltd. (Noda, Chiba, Japan), which were prepared from the liver of phenobarbital- and 5,6-benzoflavone-treated SD rats. We prepared a 10 ml S9 mix with 4 ml S9 fraction and 2 ml each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP and 150 mM KCl.

We treated 20 ml aliquots of cell suspensions (5.0×10^5 cells/ml) at 37 °C for 4 h with serially diluted AA or GA, washed them once, re-suspended them in fresh medium, and cultured them in new flasks for the MN and *TK* assays or diluted and plated them for survival measurement (PE0). We treated the cultures with AA both in the absence and presence of 5% S9 mix.

2.2. Comet assay

After treating the cells for 4 h with AA or GA, we prepared slides for alkaline COM assay as previously reported [15]. Briefly, the cells were suspended in 0.5% agarose-LGT (Nakalai Tesque Inc., Kyoto, Japan), quickly layered on a slide (Matsunami Glass Ind. Ltd., Osaka, Japan) coated with 1% agarose GP-42 (Nakalai Tesque Inc.), and covered with 0.5% agarose-LGT. We immersed the slide in alkaline lysing solution (pH 13) for 1 h, electrophoresed it for 15 min after the unwinding treatment, fixed the cells with 70% ethanol, and stained them with SYBER green (Molecular Probes, Eugene, OR) according to the manufacturer's recommendation. We observed the cells by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and the tail length of the comet image was measured. We statistically analyzed the difference between the non-treated and treated plates with the Dunnett's test after one-way ANOVA [16].

2.3. Micronuclei test

Forty-eight hours after treatment, we prepared the MN test samples as previously reported [17]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold glacial acetic acid in methanol (1:3), and resuspended in methanol containing 1% acetic acid. We placed a drop of the suspension on a clean glass slide and allowed it to air-dry. We stained the cells with 40 μg/ml acridine orange solution and immediately observed them by Olympus model BX50 fluorescence microscope. At least, 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test. The concentration–response relationship was evaluated by the Cochran–Armitage trend test [18].

2.4. TK gene mutation assay

The TK6 cell cultures were maintained for 3 days after treatment to permit expression of the *TK* deficient phenotype. To isolate the *TK* deficient mutants, we seeded cells from each

culture into 96-microwell plates at 40,000 cells/well in the presence of 3.0 μg/ml trifluorothymidine (TFT). We also plated them at 1.6 cells/well in the absence of TFT for the determination of plating efficiency (PE3). All plates were incubated at 37 °C in 5% CO_2 in a humidified incubator. The TK assay produces two distinct phenotypic classes of *TK* mutants: normally growing (NG) mutants had the same doubling time (13–17 h) as the wild type cells, and slowly growing (SG) mutants had a doubling time of >21 h. The difference is thought to be due to a putative gene near the *TK* gene. NG mutants result mainly from intragenic mutations, such as point mutations and small deletions, while SG mutants result from gross genetic changes extending beyond the *TK* gene [19]. We scored for the colonies in the PE plates and for the colonies for normal-growing *TK* mutants in the TFT plates at 14th day after plating. We then refed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing *TK* mutants. Mutation frequencies were calculated according to the Poisson distribution [20]. 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 [21].

2.5. Molecular analysis of TK mutants

Genomic DNA was extracted from *TK* mutant cells and used as a template for the polymerase chain reaction (PCR). We analyzed for loss of heterozygosity (LOH) at the human *TK* gene by PCR products as described previously [22]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that contains frameshift mutations. Another primer set for amplifying parts of the β-globin were also prepared. We used quantitative-multiple PCR to co-amplify the three regions and to identify and quantify the PCR products. We analyzed them with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and classified the mutants into "none LOH", "hemizygous LOH" or "homozygous LOH". To determine the extent of LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis described previously [22]. The results were processed by GenoTyper™ software (PE Biosystems) according to the manufacturer's guidelines.

3. Results

3.1. Cytotoxic and genotoxic responses to AA and GA

Fig. 1a shows the effect of AA on relative survival (RS), mutation frequency (*TK* assay) and number of micronucleated cells per 1000 cells examined. AA was concentration-dependently cytotoxic, permitting about 20% RS at the maximum concentration (14 mM), while its genotoxicity and clastogenicity were weak. We repeated the experiment because of the weak genotoxicity. AA showed negative in the first *TK* assay, but positive in the second statistically. In MN test, both experiments showed statistically positive. GA, in contrast, was significantly genotoxic even at concentrations that were not severely cytotoxic (Fig. 1b). At the maximum concentration (2.4 mM), GA induced *TK* mutation frequencies that were about 20 times and MN fre-

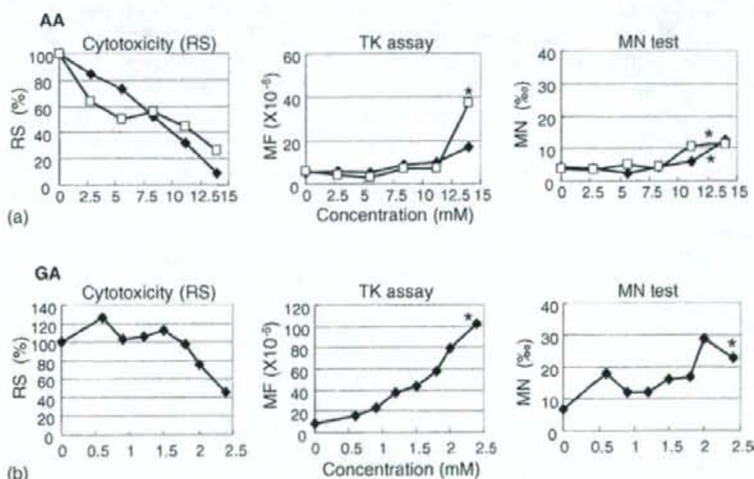

Fig. 1. Cytotoxic (relative survival, RS), genotoxic responses (TK assay and MN test) of TK6 cells treated with AA (a) or GA (b) for 4 h without metabolic activation. The AA experiment was repeated to confirm the result because of the weak genotoxicity. Closed and open symbols are first and second experiment, respectively. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

Table 1
Cytotoxic and mutational responses to AA and GA, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) TK-mutants

Treatment	Cytotoxic and mutational response			LOH analysis at TK gene			
	RS (%)	MF ($\times 10^{-6}$)	% SG	No.	None LOH	Hemi-LOH	Homo-LOH
Vehicle [16]	100	2.19	56	56			
NG mutants				19	14(74)	3(16)	2(11)
SG mutants				37	0(0)	9(24)	28(76)
AA (14 mM, 4 h)	40	18.9	54	48			
NG mutants				22	11(50)	11(50)	0(0)
SG mutants				26	0(0)	13(50)	13(50)
GA (2.2 mM, 4 h)	12	55.5	36	44			
NG mutants				28	26(93)	2(7)	0(0)
SG mutants				16	0(0)	6(38)	10(62)

quencies at about four times the spontaneous level. We detected two distinct phenotypic classes of *TK* mutants in *TK* assay: NG and SG mutants. AA did not affect the proportion of SG mutants, while GA treatment lowered it (Table 1). This implies that GA induced primarily point mutations. In the COM assay, even at the highest concentration, AA did not induce DNA damage, while GA did so strongly starting at 0.6 mM (Fig. 2).

3.2. Molecular analysis of TK mutants

The *TK* mutants were independently isolated from the cells treated with 14 mM AA or 2.2 mM GA for 4 h. Table 1 shows the cytotoxicity (RS) and *TK* mutation frequency (MF) and proportion of SG mutants (% SG) by the treatment. Genomic DNA extracted from the mutants was subjected by the PCR-based LOH analysis to classify the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH) and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [13]. We analyzed 48 AA-induced and 44 GA-induced *TK* mutants and compared them to those of spontaneously occurring *TK* mutants described previously [16]. The fraction of hemi-LOH in AA-induced mutants, in which 50% each of NG and SG mutants exhibited hemi-LOH, was higher than in spontaneous mutants, indicating that AA-induced primarily deletions. GA, on the other hand, induced primarily NG mutants, and most (93%) of them were the non-LOH type, which is presumably generated by point and other small intragenic mutations. Among 16 GA-induced SG mutants, the percentages that were hemi-LOH (38%) and homo-LOH (62%) were similar to those observed in spontaneous SG mutants. Fig. 3 shows the mutation spectra of *TK* mutants found among treated and untreated TK6 cells. GA and ethyl methane sulfonate, an alkylating agent, produce similar spectra, as do AA and X-radiation.

Fig. 4 shows the distribution of LOH in AA-induced ($n = 37$), GA-induced ($n = 17$) and spontaneous ($n = 29$) LOH mutants. Because the majority of GA-induced mutants were the non-LOH type, we were able to map only 17 GA-induced LOH mutants. As a particular characteristic of AA-induced LOH mutants, we frequently observed small deletions limited to the *TK* locus. The

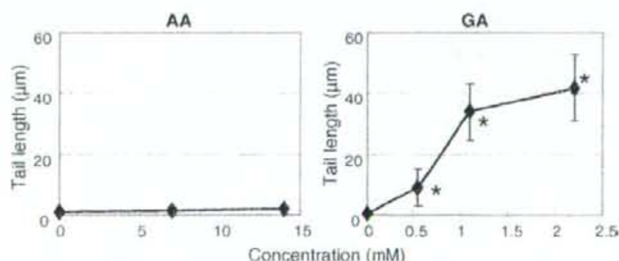

Fig. 2. COM assay results in TK6 cells treated with AA or GA for 4 h without metabolic activation. Asterisk (*) statistically significant in the Dunnett's tests ($P < 0.05$).

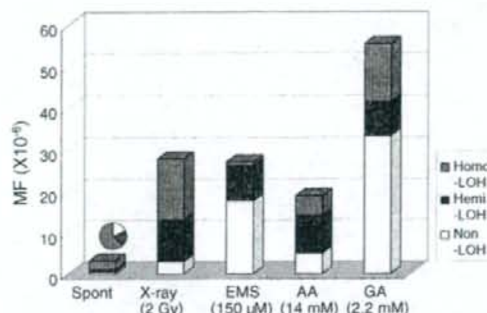

Fig. 3. Frequency and spectra of TK mutations in spontaneous and X-ray-induced (2 Gy), EMS-induced (150 μM, 4 h), AA-induced (14 mM, 4 h) and GA-induced (2.2 mM, 4 h) TK mutants in TK6 cells. The fraction of each mutational event was calculated by considering the ratio of normally growing (NG) and slowly growing (SG) mutants and the results of molecular analysis (Table 1). The data of spontaneous, X-ray-induced and EMS-induced mutation spectra were taken from our previous paper [13].

distribution of LOH in GA-induced and spontaneous LOH mutants was similar.

3.3. Cytotoxicity and genotoxicity of AA under metabolic activation

Rat liver S9 mix did not influence the cytotoxicity or genotoxicity of AA but it did enhance the activity of DBN, the positive control chemical (Fig. 5).

4. Discussion

A large number of studies about the in vitro genotoxicity of AA have been reported [8]. AA has consistently been negative in bacterial gene mutation assay in both the presence and absence of metabolic activation [23–25] but positive in chromosome aberration and sister chromatid exchange tests in Chinese hamster cell lines [24–26]. In mammalian cell assays, AA induces *Tk* but not *Hprt* gene mutations [24,25,27,28], and is negative in the COM assay even at high concentrations [27]. These results suggest that AA is clastogenic without directly damaging DNA. GA, on the other hand, is positive in most in vitro genotoxicity tests and is recognized as a mutagen [8,27,29]. In the present study, the higher concentrations of AA were positive in the MN and TK assay but negative in the comet assay. According to the in vitro genotoxicity test guideline, however, AA may be negative [30], because the guideline suggests that the maximum concentration should be 10 mM. Because the genotoxic responses at higher concentrations were reproducible, AA may be genotoxic, but its effect is very weak. GA, in contrast, was positive in all the assays, even under conditions of low cytotoxicity. These results are consistent with the reports described above.

The mammalian *TK* gene mutation assay can detect a wide range of genetic changes, including point mutations, small deletions, large-scale chromosomal deletions, inter-allelic recombination and aneuploidy, while

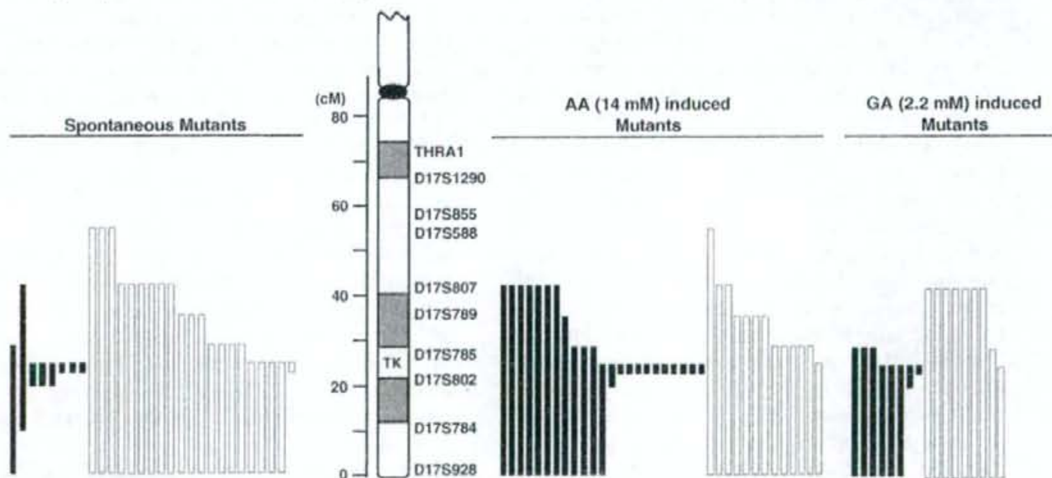

Fig. 4. The extent of LOH in spontaneous, AA-induced and GA-induced LOH mutants from TK6 cells. We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human TK locus maps to 17q23.2. Open and closed bars represent homo-LOH and hemi-LOH, respectively. The length of the bar indicates the extent of the LOH. We analyzed 29 spontaneous mutants (10 NG and 19 SG mutants), 37 AA-induced mutants (11 NG and 26 SG) and 17 GA-induced mutants (2 NG and 15 SG). The data on spontaneous mutants were taken from our previous paper [13].

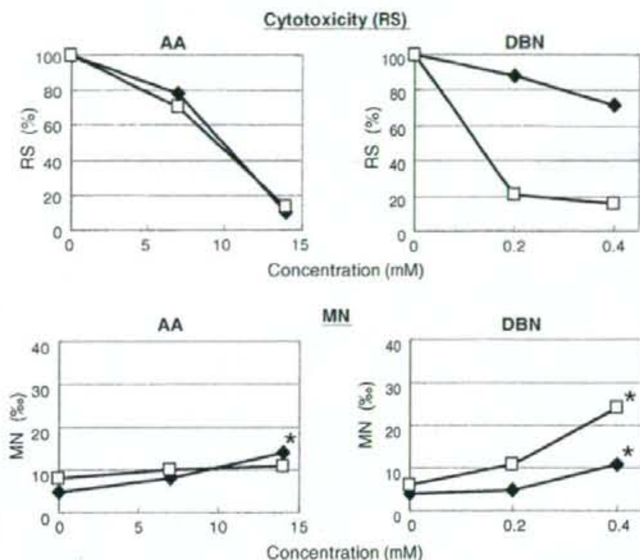

Fig. 5. Cytotoxicity (RS) and MN induction in TK6 cells treated with AA or DBN for 4 h in the presence (open symbol) or absence (closed symbol) of rat liver S9. Asterisk (*) statistically significant experiments in both pair-wise comparison and trend test ($P < 0.05$).

the bacterial and mammalian *HPRT* gene mutation assays detect only point mutations and small intragenic deletions [13]. AA was positive only in the *TK* mutation assay, suggesting that AA causes predominantly large-scale chromosomal changes. Our molecular analysis of the *TK* mutants supported this hypothesis. The majority of the AA-induced *TK* mutants showed hemi-LOH, which is the result of a deletion, although the other types were also induced (Fig. 3). Deletions are thought to result from the repair of double strand breaks by non-homlogous end-joining [13]. Radiation-induced double strand breaks are repaired by non-homlogous end-joining, which leads to hemi-LOH. LOH-mapping analysis, however, revealed that AA frequently induces intermediate-sized deletions (100–3000 kb); the deletions encompass exons 4 and 7 of the *TK* locus but do not extend to the microsatellites loci of the vicinity. This type of deletion is rarely observed in radiation-inducing *TK* mutants [13]. Because the COM assay indicated that AA did not induce DNA damage, the deletion may not be caused by DNA damage directly. Mechanisms associated with global genomic instability should also be considered [10] because the LOH patterns, except for the intermediate-sized deletions, are generally similar to those observed in spontaneous mutants. Most GA-induced *TK* mutants, on the other hand, were the non-LOH type, as were most spontaneous ones, strongly supporting the positive results in bacterial gene mutation assay [29]. In contrast to AA, GA is a mutagen, inducing primarily point mutations.

AA is known to be metabolized to GA by CYP2E1 [9]. GA, an epoxide, forms adducts directly with DNA and protein, causing cytotoxicity and genotoxicity. GA forms mainly *N*7-(2-carbamoyl-2-hydroxyethyl) guanine and *N*3-(2-carbamoyl-2-hydroxyethyl) adenine and reacts with hemoglobin and cytoskeletal proteins [31–33]. Rat S9, however, did not affect AA cytotoxicity or genotoxicity, although it did enhance the cytotoxicity and genotoxicity of DBN, which is also metabolized by CYP2E1. This suggests that rat S9 does not work for activating AA. AA and GA are detoxified through glutathione conjugation, and GA is also detoxified by epoxy hydrolase (EH), which catalyzes the hydrolysis of GA to dihidroxy propionamide [34,35]. Other in vitro studies also failed to demonstrate the enhancement of AA genotoxicity by rat S9 [36,37]. Our results do not mean that AA is always detoxified rather than activated because DNA adducts are found in mice and rats given oral AA, and the genotoxicity of AA is consistently observed in in vivo studies [8,31,36,37]. Recently, Manjanatha et al. demonstrated in transgenic Big Blue^{TM} mice that AA as well as GA induces endogenous *Hprt* and transgenic *cII* mutation at same level, and both chemicals cause predominantly base substitutions and frameshift mutations.

This result may indicate that AA is metabolized to GA in vivo [38]. Tests that use rat liver S9 for metabolic activation may not be appropriate for in vitro investigations of AA genotoxicity and metabolism. Transgenic cells expressing CYP2E1, however, would be useful for demonstrating the in vitro genotoxicity of AA [39].

In conclusion, AA is weakly genotoxic, causing chromosome aberrations and a type of genomic instability. GA, its epoxide metabolite, is highly reactive with DNA. GA is a strong mutagen, inducing predominantly point mutations, and it may contribute to human cancers.

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