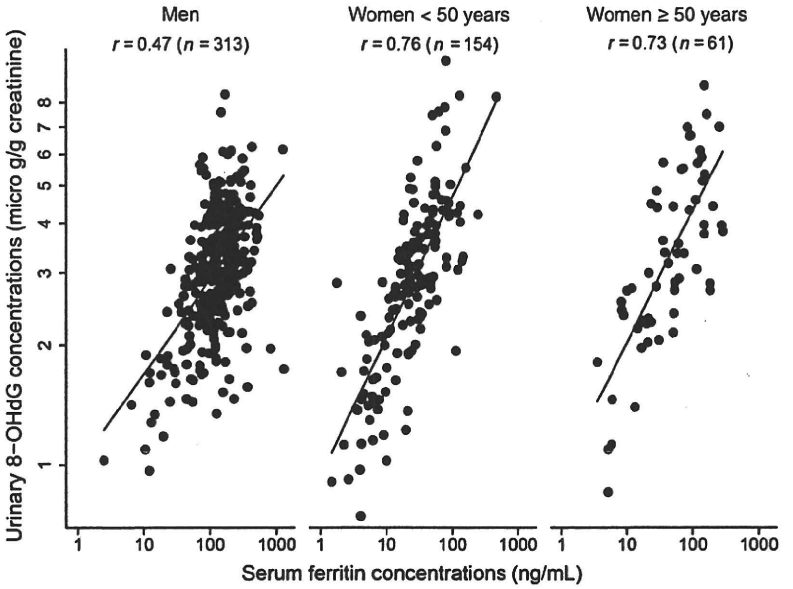

Fig. 1. Correlation between serum ferritin and urinary 8-hydroxydeoxyguanosine (8-OHdG) concentrations.

tertile of ferritin among women aged less than 50 years (1.80 μg/g creatinine). Urinary 8-OHdG concentrations were not significantly associated with red blood cell count, dietary iron intake, and serum iron concentrations (data not shown).

Discussion

In this cross-sectional biomarker study of healthy Japanese men and women, we found a strong positive association between serum ferritin and urinary 8-OHdG concentrations. This association was robust in all the subgroups whose serum ferritin concentrations markedly differed, and were materially unchanged even after adjustment for potential confounding variables. Our finding underscores the importance of body iron store as a determinant of level of oxidative DNA damage in a population without iron metabolism disorders.

8-OHdG, an oxidized nucleoside of DNA, is the most frequently detected DNA lesion in nuclear and mitochondrial DNA. Several reactive oxygen species, such as hydroxyl radical and singlet oxygen, attack on C-8 of guanine in DNA and generate 8-OHdG. 8-OHdG and its oxidation products lead to GC → TA transversions in human cells since 8-hydroxyguanine has hydrogen-bonding ability to adenine.[3] Base excision repair by 8-hydroxyguanine glycosylase1 is the major removal path of 8-OHdG from DNA.[1] Removed 8-OHdG from the whole body is excreted in human urine,[29] and thus urinary 8-OHdG is considered to reflect oxidative DNA damage and repair from all cells in the organism.

Ferritin is an iron binding protein that can store up to 4500 Fe^{3+} ions and is distributed throughout the body, and its concentrations in serum reflect the body's iron store.[30] Although ferritin itself has the potential to protect against oxidative stress by chelating free iron,[31] it could also act as a mediator of oxidative stress by releasing free iron.[32] This paradoxical behavior makes the role of ferritin in oxidative stress controversial.[33]

Previously, two studies have examined the association between serum ferritin and 8-OHdG concentrations in persons with no known condition that might influence these measurements. In a study of mild dyslipidemic men,[21] there was a significant positive association between the two measures (Spearman rank correlation coefficient, 0.36). However, this finding is limited due to the small sample size (n = 48) and

Table 2. Urinary 8-OHdG concentrations by serum ferritin levels

	Ferritin tertile (ng/mL)	Geometric mean (95% CI) of 8-OHdG concentrations (μg/g creatinine)						
		n	Unadjusted	Model 1†	*n*	Model 2‡	*n*	Model 3§
Women <50 years	<17	51	1.80 (1.61–2.00)	1.90 (1.65–2.20)	51	2.16 (1.82–2.56)	51	1.91 (1.63–2.24)
	17–35	51	2.93 (2.69–3.19)	3.04 (2.60–3.56)	51	3.20 (2.73–3.75)	51	3.00 (2.52–3.58)
	≥36	52	4.19 (3.82–4.61)	4.37 (3.76–5.08)	52	4.57 (3.92–5.33)	49	4.39 (3.71–5.19)
	P for trend		<0.001	<0.001		<0.001		<0.001
Women ≥50 years	<25	20	2.03 (1.69–2.43)	1.66 (1.16–2.37)	20	1.84 (1.22–2.76)	20	1.44 (1.01–2.04)
	25–84	20	3.57 (3.05–4.19)	2.87 (1.91–4.30)	20	3.11 (2.02–4.77)	20	2.24 (1.47–3.39)
	≥85	21	4.87 (4.21–5.64)	3.92 (2.49–6.16)	21	4.19 (2.64–6.64)	21	3.41 (2.21–5.28)
	P for trend		<0.001	<0.001		<0.001		<0.001
Men	<98	105	2.47 (2.30–2.65)	2.47 (2.32–2.63)	99	2.38 (2.21–2.56)	105	2.45 (2.30–2.61)
	98–179	102	3.22 (3.03–3.43)	3.17 (2.98–3.38)	97	3.05 (2.83–3.28)	98	3.24 (3.03–3.47)
	≥180	106	3.56 (3.36–3.77)	3.59 (3.35–3.83)	98	3.39 (3.13–3.68)	106	3.58 (3.34–3.84)
	P for trend		<0.001	<0.001		<0.001		<0.001

†Adjusted for age, smoking status, and body mass index. ‡Adjusted for age, smoking status, body mass index, and hemoglobin. §Adjusted for age, smoking status, body mass index, alcohol consumption, physical activity, vitamin C and vitamin E intake. 8-OHdG, 8-hydroxydeoxyguanosine; CI, confidence interval.

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subjects' profile (dyslipidemic men), which may not represent the general population. In another study in a health checkup setting,[18] the correlation coefficient between serum ferritin and urinary 8-OHdG concentrations were 0.32 and 0.54 for men and women, respectively. However, the source population of the health checkup attendants was not clearly defined and no consideration was made for potentially important confounders including smoking. The observed association in our study (Spearman rank correlation coefficient, 0.47 and 0.76 for men and women, respectively) appears stronger than those documented previously. Our study participants shared a similar social background (municipal employees) and the survey was conducted in a short period of time (less than 10 days for each survey). We believe that such study design might have contributed to the minimization of the confounding effect of unmeasured variables.

Epidemiologic studies have reported an increased cancer risk associated with menopause[34] or high body iron status[35] in women. In the present study, women aged 50 years or older had markedly higher ferritin concentrations than did women aged less than 50 years, whereas hemoglobin levels did not differ. Although we did not obtain information regarding menopausal status from the study participants, given that the human body does not have a specific excretion route for stored iron[36], this probably reflected an increase of body iron storage after menopause. Moreover, in accordance with the difference of ferritin concentrations, women aged 50 or older had significantly higher 8-OHdG levels than women aged less than 50 years, suggesting an increased oxidative DNA damage after menopause. Because the regression line of serum ferritin concentrations on urinary 8-OHdG levels was similar between the two women's age groups, the increased levels of oxidative DNA damage among the older women could be ascribed to their elevated body iron storage, rather than to decreased physiologic function against oxidative stress after menopause.

Several epidemiologic studies have shown an increased risk of cancer associated with high dietary intake of heme iron[5,6] and high blood levels of iron.[7–10] Moreover, as a phlebotomy intervention study indicated,[11] iron reduction may decrease cancer risk even among persons without iron metabolism disorders. Together with accumulating data for the usefulness of urinary 8-OHdG as a marker of cancer risk,[15–17] the strong positive association between serum ferritin concentrations and urinary 8-OHdG concentrations in the present study supports the hypothesis that body iron storage increases cancer risk through oxidative DNA damage in humans.

Major strengths of the present study include high participation rate (91%), adjustment of potential confounding variables in the analysis, and use of a reliable method for 8-OHdG measurement (HPLC). Our study has also some limitations. First, causality can not be inferred from any cross-sectional study, like ours. Second, we measured biomarker levels only at a single point in time, which may not represent long-term status. Third, other biomarkers of body iron including transferrin saturation or soluble transferrin receptor[37] were not measured in the present study. However, ferritin is considered a preferred marker for the assessment of iron-related oxidative stress.[38] Finally, the study subjects were healthy municipal workers, and thus caution should be exercised when applying the result to populations with a different background.

In conclusion, we found a strong positive association between urinary 8-OHdG levels and serum ferritin concentrations in Japanese workers. This finding suggests that body iron storage is an important determinant of oxidative DNA damage, and thus supports a significant role for iron in carcinogenesis in a general population. The observed cross-sectional association requires confirmation in longitudinal studies.

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Research Article

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

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The recent finding that acrylamide (AA), a genotoxic rodent carcinogen, is formed during the frying or baking of a variety of foods raises human health concerns. AA is known to be metabolized by cytochrome P450 2E1 (CYP2E1) to glycidamide (GA), which is responsible for AA's in vivo genotoxicity and probable carcinogenicity. In in-vitro mammalian cell tests, however, AA genotoxicity is not enhanced by rat liver S9 or a human liver microsomal fraction. In an attempt to demonstrate the in vitro expression of AA genotoxicity, we employed *Salmonella* strains and human cell lines that overexpress human CYP2E1. In the *umu* test, however, AA was not genotoxic in the CYP2E1-expressing *Salmonella* strain or its parental strain. Moreover, a transgenic human lymphoblastoid cell line overexpressing CYP2E1 (h2E1v2) and its parental cell line (AHH-1) both showed equally weak cytotoxic and genotoxic responses to high (>1 mM) AA concentrations. The DNA adduct N7-GA-Gua, which is detected in liver following AA treatment in vivo, was not substantially formed in the in vitro system. These results indicate that AA was not metabolically activated to GA in vitro. Thus, AA is not relevantly genotoxic in vitro, although its in vivo genotoxicity was clearly demonstrated. Environ. Mol. Mutagen. 52:12–19, 2011.

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

umu Assay

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

Mammalian Cell Assays Measuring Gene Mutation and Chromosome Damage

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

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

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

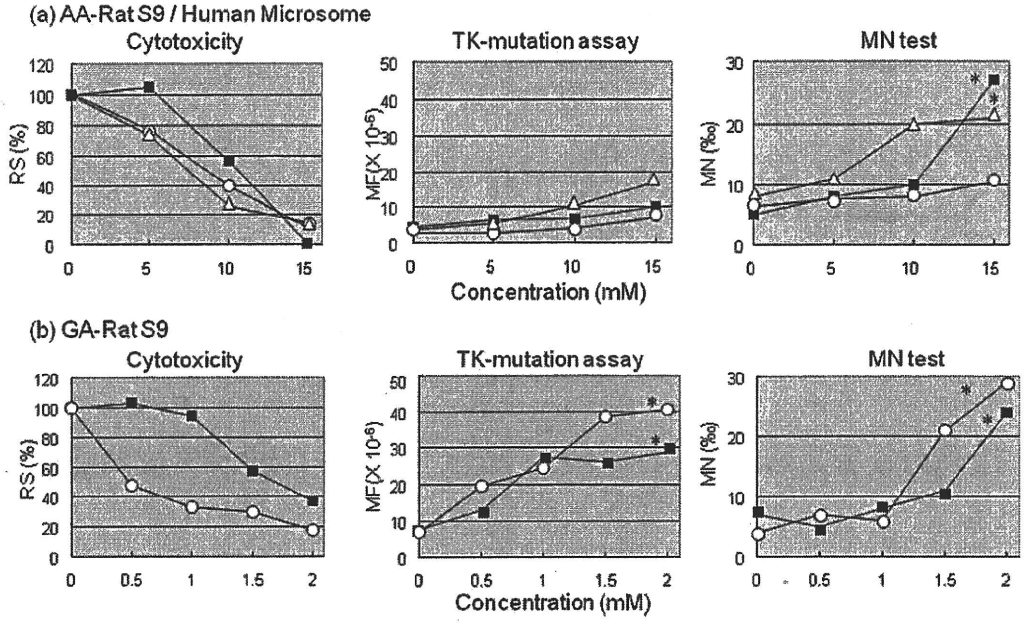

Fig. 1. Cytotoxic (relative survival, RS) and genotoxic (TK and MN assays) responses of TK6 cells treated with AA or GA for 4 hr with or without metabolic activation. (a) TK6 cells were treated with AA without (■) or with (○) rat liver S9 or human microsomes (△). (b) TK6 cells were treated with GA without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

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

Western Blot Analysis

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

DNA Adduct Assay

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

RESULTS

Cytotoxicity and Genotoxicity of AA and GA Under Metabolic Activation

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

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

umu Assay Using Strains Expressing Human CYP2E1

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

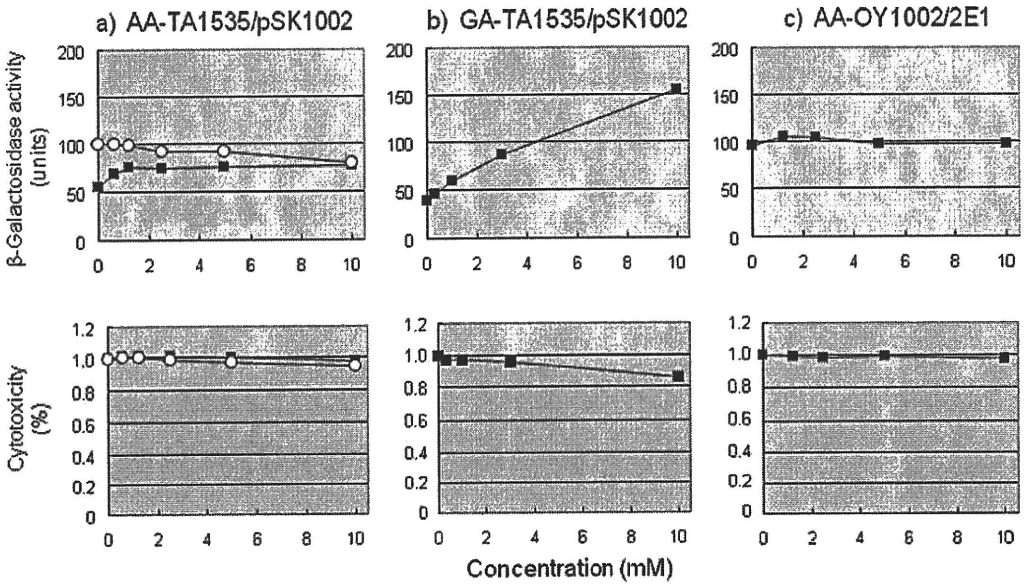

Fig. 2. Induction of *umuC* gene expression and cytotoxic response by AA (a, c) or GA (b) in *S. typhimurium* tester strains TA1535/pSK1002 (a, b) and OY1002/2E1 (c). The *umu* tests were conducted without (■) or with rat S9 (○). β-Galactosidase activity (units) was determined as described in Materials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

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

Cytotoxic and Genotoxic Responses to AA in Transgenic Cell Lines

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

DNA Adduct Formation by AA and GA in the Cell Lines

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

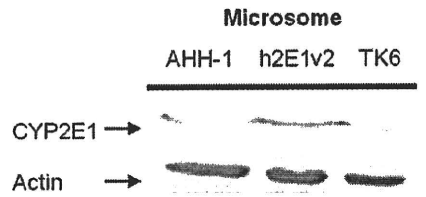

Fig. 3. Western blot analysis of CYP2E1 in AHH-1, h2E1v2, and TK6 cells. Equal amount of materials were loaded for each sample. CYP2E1 protein was stained with the anti-CYP2E1 antibody. Actin was used as a loading control.

DISCUSSION

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

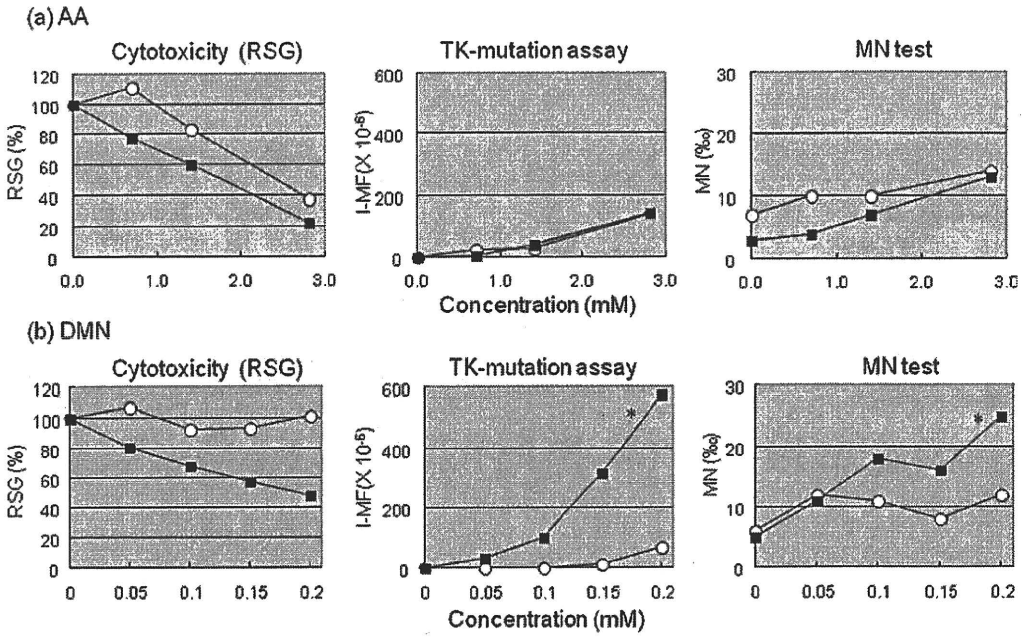

Fig. 4. Cytotoxic (relative suspension growth, RSG) and genotoxic (TK assay and MN test) responses of AHH-1 (○) or h2E1v2 (■) cells treated with AA or DMN for 4 hr. I-MF means induced mutation fraction, in which back ground mutation frequency is subtracted. $*P < 0.05$ (Omori method for TK-mutation assay,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

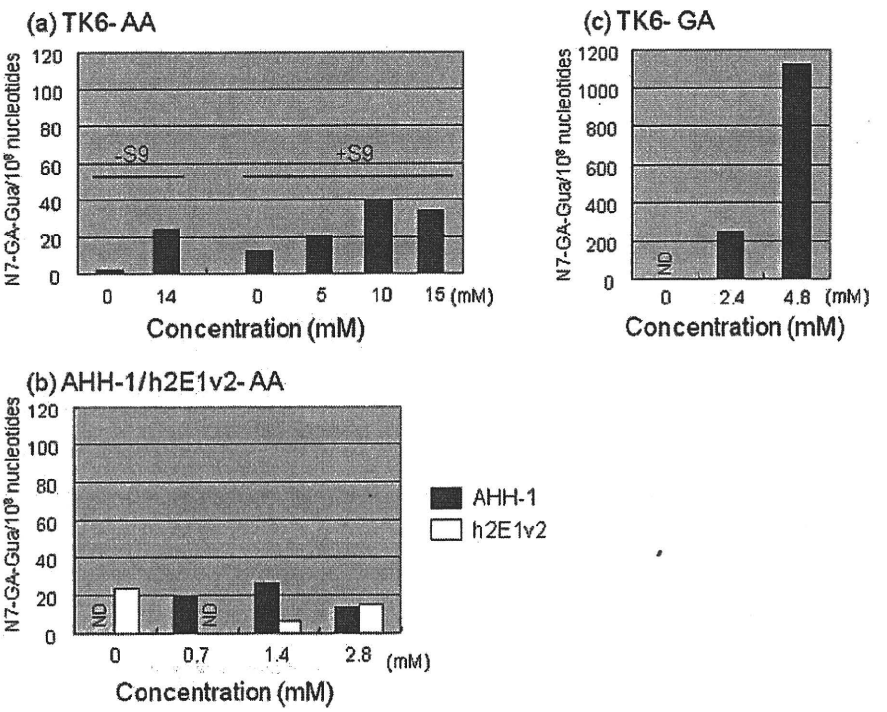

Fig. 5. Levels of N7-GA-Gua adduct in TK6 (a, c), AHH-1 (b), or h2E1v2 (b) cells treated with AA (a, b) or GA (c) for 4 hr at different concentrations. Data are expressed as the number of adducts in 10^8 nucleotides.

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

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

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

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

The *umu* assay could not detect the genotoxicity of AA even by the strain (Fig. 2). Emmert et al. [2006] also failed to demonstrate the mutagenicity of AA in the Ames test using the metabolically competent *S. typhimurium strain* YG7108pinERb_5 that expresses CYP2E1. In mammalian cell system, such as the human lymphoblastoid cell line, h2E1v2 overexpressing human CYP2E1 did not show different response in *TK*-gene mutation and MN induction compared to its parental cell line, AHH-1, although these cell lines exhibited distinct difference to DMN, which is a representative substrate for CYP2E1. We also investigated the genotoxicity of AA in h2E1v2 cells after long exposure (24 hr), because AA may be slowly metabolized to GA. The result was also negative (data not shown). Thus, we could not obtain any evidence of in vitro genotoxicity of AA *via* metabolic activation.

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

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

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

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

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解　説
Review

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各種ナノ粒子の細胞への影響：細胞特異性とその応用

Effects of Various Nanoparticles on Human Cells: Cell Specificity and its Application

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Application fields of nanoparticles range from biomedicine such as imaging and drug delivery systems to various industrial products. Despite the rapid progress, the potential for nanotoxicity in human has not yet been established. Most methods for toxicity assessment were designed and standardized with chemical toxicology. The reliable toxicity test systems are needed. Here, we present an overview of current *in vitro* toxicity tests for nanoparticles risk assessment, and focus on genotoxicity, especially cell-specific genotoxicity and its application.

Keywords: Nanoparticle, Cytotoxicity, Genotoxicity, Cell specificity

1．緒　言

ナノテクノロジーは，ナノスケール（10億分の1メートル）の超微小な領域において，物質を取り扱う技術である。ナノスケールは，例えばインフルエンザウィルスや筋肉を構成するアクチン，ミオシンタンパ

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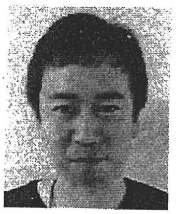

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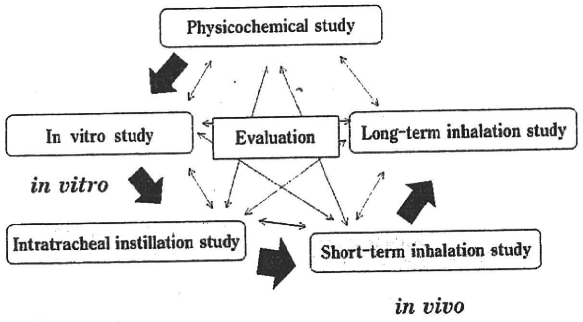

Fig. 1 Hazard assessment system for nanoparticles

ク質と同程度の大きさで，これらと比較しても扱う技術の超微細さが理解できる。2000年1月にクリントン米国大統領によって出された教書「国家ナノテクノロジー優先施策（National Nano-technology Initiative: NNI）」でも示されたように，ナノテクノロジーは素材，バイオ，医療などの産業の基盤にかかわるものであり，21世紀の最重要な技術の一つであるのは周知の事実である。ナノテクノロジーを支える重要なものであるナノ材料の研究開発は，これまでのバルク体に無い，サイズの縮小による細胞・組織への浸透性の増大，表面活性と反応性の向上，電磁気的，光学的，機械的などの物理化学的特性から，その応用が注目を浴びている。これらのナノサイズの特性を利用して，ナノ酸化チタン，フラーレン，ナノシルバー等が化粧品，スポーツ用品等に使用されている。医療分野では，ドラックデリバリーシステム（DDS）や，生体組織構築技術などの応用研究が行われている。健康で安心・安全な社会を実現するため，ナノ材料はなくてはならないものと考えられる。しかしながら，同じ材質であっても，バルク体とは異なる特性を示すため，ナノ材料の生体への曝露あるいは環境放出による影響が問題となる。そのため，新規化学物質と同様に，ナノ材料のリスク評価・リスク管理が求められている。経済産業省の「ナノ粒子特性評価手法の研究開発」（平成18～22年度）をはじめとして，厚生労働省等が有害性試験の結果の発表，報告を行っている[1,2]。ナノ材料の人へのリスク評価は，ナノ材料のキャラクタリゼーションの後にナノ材料の有害性（ハザード）と同材料の人間への曝露量と曝露経路の両面から評価される。有害性の評価として，*in vitro* 評価および *in vivo* 評価がある（**Fig. 1**）[3]。ナノ材料の吸入曝露試験がヒトへの曝露状態を模擬している曝露手法で，生体影響のデータの信頼性が高い。吸入曝露試験よりは劣るが，気管内注入試験により吸入曝露試験の代替や予備試験として行われるが，既知量による用量反応関係を調べることができるので，一般的には有害性評価に有用な試験である。

ナノ材料の有害性の評価は，従来の化学物質の評価と同様に行い得ないのが重要なことである。*in vivo* あるいは *in vitro* 実験系において，ナノ粒子では凝集体として考えられる2次粒子として評価されることで，本来の1次粒子（ナノサイズ）の影響を評価していない等のいろいろな問題を抱えている[2,4]。

本稿では，ナノ材料，特にナノ粒子の細胞毒性および遺伝毒性を評価する *in vitro* 試験についての紹介，これら *in vitro* 試験より得られた知見から示唆されたナノ粒子の細胞特異性について紹介する。

2．細胞毒性試験について

ナノ粒子の有害性評価のために，*in vitro* 試験がある。これは，培養細胞にナノ粒子を曝露させ，細胞への影響を観察する細胞毒性試験である。培養細胞にナノ粒子を曝露させると，濃度勾配による拡散，Receptor mediated endocytosis, Caveolae-mediated endocytosis, Clathrin-mediated endocytosis 等の機序により，各ナノ粒子は細胞内に入り込む[5]。使用される細胞は様々であるが，特に肺胞マクロファージとして RAW 264.7（Mouse leukaemic monocyte macrophage cell line）や，肺胞上皮細胞として A549（Human lung adenocarcinoma epithelial cell line）等の肺癌細胞株が使用される。これらは，正常細胞の培養の難しさ，ナノ粒子の呼吸器系を介した曝露を想定した場合に，最初に遭遇し，肺組織での細胞－ナノ粒子の相互作用が生じると考えられることや *in vivo* 実験を考えた上で選択される。細胞毒性試験の評価法を，**Table 1** に示す[6]。ナノ粒子の吸着能や光学的性質等の特性がこれら試験に影響を与えていることも考えられており，注意が必要である。

細胞生存率（Cell vaiability）は細胞毒性試験の最も一般的に調べられる内容であるが，異なる測定エンドポイントが利用されている。

2．1 MTT assay

テトラゾウリウム塩のミトコンドリア内膜内脱水素酵素による還元を伴うホルマザン色素の呈色反応を利用するアッセイである。色々改良され，現在では細胞毒性評価に多用され，他の評価法の検証にも使用されている。呈色反応に金属イオン，pH が影響するなど報告され，また，基質とナノ粒子が反応する等の問題が報告されている[6]。

Table 1 Current *in vitro* cytotoxicity assays

Cytotoxicity assay	Detection principle
MTT	Colorimetric detection of mitochondrial activity
LDH	Colorimetric detection of LDH release
Annexin V/Propidium Iodide	Fluorimetric detection of Phospatidylserine exposure (Apoptosis marker)/Propidium Iodide-staining of DNA (Necrosis marker)
Neutral red	Colorimetric detection of intact lysosome
Caspase-3	Fluorimetric detection of Caspase-3 activity (Apoptosis marker)
H_2DCF-DA	Fluorimetric detection of ROS production
ELISA	Colorimetric detection of cytokine secretion

2. 2 Lactate Dehydrogenase (LDH) 活性測定

生細胞に存在するLDHで，細胞膜の損傷に伴い細胞外へ遊離したLDHの活性を測定する。LDH活性測定も，pHや金属イオンの影響を受けると報告されている[6]。

2. 3 Annexin/propidium iodideを利用した測定

正常では脂質2重層の内側に存在する負電荷をもったphosphatidylserine (PS) が，apoptosisの初期に外側に転移する現象がみられる。Annexin VはCa^{2+}存在下でPSに対して強い親和性をもつため，Annexin VはPSが細胞表層に露出したapoptosis細胞のみに結合し，apoptosisの検出が可能となる。necrosis細胞もPSが露出するので，necrosis細胞とapoptosis細胞の鑑別のため，色素排除テストに使用されるDNA染色剤としてのPropidium Iodideを同時使用する。

2. 4 Neutral red assay

Neutral red (3-amino-7-dimethyl-amino-2- methyl phenazine hydrochloride) は可溶性の色素で，生細胞のみ細胞内に取り込まれる。細胞に取り込まれたNeutral redを洗浄後に抽出して540nmの吸光度で測定する。

2. 5 Caspase-3の検出

活性型Caspase-3の検出は最も一般的に使用されるapoptosis assayである。活性型Caspase-3は発色基質あるいは蛍光基質の分解物を比色（分光光度計）または蛍光（フルオロメーター）で測定することにより検出される。Caspase-3は金属イオン，特にZn^{2+}により阻害される[6]。

2. 6 活性酸素種 (Reactive Oxygen Specimens : ROS) の検出

H_2DCF-DAは，細胞内のROS検出のための蛍光プローブとして広く使用されている。生細胞の細胞質で酸化されるまでは，細胞透過性の非蛍光物質である。生細胞に入り，ROSが存在すると，還元された色素が酸化され，色素が蛍光を発する。

2. 7 Enzyme-linked immunosorbent assay (ELISA) による炎症マーカーの検出

Tumor necrosis factor alpha (TNF-α) やInterleukins等がELISAにより検出される。

3．遺伝毒性評価について

ナノ粒子の安全性評価に関する論文の多くは，細胞毒性に関する内容である。しかし，ナノ粒子もDNAに障害を及ぼし，癌や遺伝性疾患を引き起こす可能性が考えられ，細胞毒性と同様に評価をしなければならない。

自然突然変異よりも高頻度で，突然変異を誘発する物理的，化学的，生物的要因の総称を環境変異原と呼ぶ。環境中には多種多様な化学物質があり，突然変異を誘発する化学変異原も少なくないことから，一般に変異原というと化学変異原をさす場合が多い。当初は，変異原物質と遺伝毒性物質は同意であった。その後，変異原はDNA損傷性を除く，遺伝子突然変異あるいは染色体異常を誘発する物質に対して用いられている。(日本環境変異原学会関連用語の解説 http://www.j-ems .org/info/glossary.html#ha)

遺伝毒性試験には，色々な機構で遺伝的な傷害を引き起こす物質を検出するための *in vitro* および *in vivo* 試験がある。これらの試験は，遺伝物質に対する毒性，すなわちDNA損傷性から突然変異誘発性，染色体異常誘発性までを検出する。これらの意義は，後世代への遺伝性影響であり，また，発癌への関連性と考えられている。すなわち，遺伝毒性試験陽性の物質は，変異原物質や発癌物質である可能性があるが，また必ずしも変異原物質が発癌物質ではない。いずれにせよ，ナノ粒子の遺伝毒性と発癌性の評価は，重要な

Table 2 Current genotoxicity assays

Genotoxicity assay	Detection principle
Ames test	The application of mutagen leads mutations in many genes including the defective gene and some of those mutations cause the reversal of ability to synthesize histidine.
Chromosome aberration test	After exposure of cells to the test substance, they are treated with a metaphase-arresting substance, harvested, stained and metaphase cells are analysed microscopically for the presence of chromosome aberrations.
Mouse lymphoma test	Mutant cells, deficient in TK due to the forward mutation in the TK locus, are resistant to the cytotoxic effect of pyrimidine analogues such as trifluorothymidine.
Micronucleus test	Detection of chemicals which induce the formation of small membrane bound DNA fragments i.e. micronuclei in the cytoplasm of interphase cells
Comet assay	Measuring DNA single- and double-strand breaks at the level of individual cells
8-OHdG	Detection of 8-OHdG formation

課題である。遺伝毒性試験については，**Table 2** に示す[7,8]。

3. 1 微生物を用いる復帰突然変異試験（Ames 試験）

本試験は，1972年に Ames 博士らにより変異原性を評価するために開発された。サルモネラ菌を用い，ヒスチジン（histidine）要求性株が変異を起こし，ヒスチジン非要求株になり，寒天培地上でのコロニー数の変化を復帰突然変異として評価する。Ames 博士は，化学物質の持つ発がん性と変異原性の関係を明らかにしたなどの功績で，杉村博士とともに1997年の日本国際賞を受賞した。この他，大腸菌を用いて，トリプトファン（tryptophan）要求性株が非要求性株になることを評価する。これら細菌を利用する復帰突然変異試験では，塩基対置換変異，フレームシフト変異などの点突然変異が調べられる。Ames 試験は，簡便性，経済性および効率性に優れている。しかしながら，原核生物のデータを真核生物の評価へ移し替えることの難しさがある。陽性であっても発癌性のない物質，陰性であっても発癌性のある物質があることも周知の事実である。

3. 2 ほ乳類動物細胞を用いる染色体異常試験

本試験は，チャイニーズハムスター肺細胞やヒト末梢血リンパ球を用いて，染色体の構造異常や数的異常を評価する。有害物質の曝露された細胞は，染色体を構成する DNA やタンパク質が障害を受けて，細胞分裂中期の染色体異常として観察される。本試験が陽性でも発癌性は示さない場合もあることや，cytology の技術が必要とされる。労働安全衛生法，化学物質審査規制法，薬事法などの法規制で実施が義務付けられている重要な試験である。

3. 3 Mouse Lymphoma 試験

本試験は，マウスリンパ性白血病由来 L5178Y を用いて，チミジンキナーゼ *tk* 遺伝子上に生じる変異の有無を評価する。L5178Y 細胞はヘテロに *tk* 遺伝子をもち（*tk*+*l*−），変異の誘発（*tk*−*l*−）によりチミジンキナーゼの代謝拮抗剤，トリフルオロチミジンへの耐性変異コロニーとして観察される。本試験は，点突然変異から染色体レベルの欠失変異も捉えられることやほ乳類細胞を用いることで，Ames 試験より高次な試験と考えられる。

3. 4 Micronucleus Test（小核試験）

本試験は，げっ歯類に物質を投与して，その赤血球における小核形成を評価する。赤芽球の分裂成熟過程で，染色体の異常が誘発され，小核が形成され，脱核後に細胞質に取り残された小核を観察する。本試験は，*in vitro* 遺伝毒性試験で検出しにくい物質や *in vitro* 遺伝毒性試験で陽性になった物質の *in vivo* 評価のために実施される。

3. 5 Comet assay（単細胞ゲル電気泳動法）

本試験は，電気泳動によりアガロースゲル中で DNA を移動させることにより，単細胞レベルでの1本鎖 DNA 切断や2本鎖 DNA 切断を検出し，また，その切断量に基づき修復動態を評価できる[9]。この泳

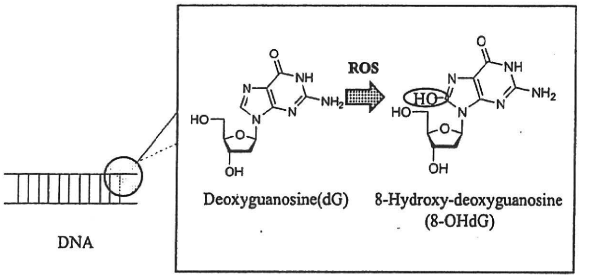

Fig. 2 Structure of 8-OHdG

動像はコメットに似て，そのテールは1本鎖DNA切断量と連動し，多くの遺伝毒性物質が1本鎖DNA切断を生じるので，非常に感度が高く，簡便で，偽陽性率の低い方法である。近年非常に使用されることが多くなっている。

3. 6 8-hydroxydeoxyguanosine (8-OHdG) 測定

8-OHdGはDNAを構成する塩基の一つであるdeoxyguanosine（dG）の8位がヒドロキシル化されたものであり，DNA酸化損傷の指標の一つである（**Fig. 2**）。1984年に葛西らにより報告されて以来，生物学的重要性，疾患との関連性が明らかにされてきた。特に，8-OHdGはDNA複製時にGC-TAのtransversion型の変異を引き起こすことより，密接に発癌に関与する。一方，ヌクレオチドプール中のDNA合成基質が酸化的損傷を受けた時も，DNA複製の際に誤った塩基が取り込まれることや，DNA中の8-OHdGを取り除くhOGG1や，ヌクレオチド中に存在する8-OHdGTPを浄化するMTH1などの修復機構も含めて，評価する必要がある[10]。8-OHdGの分析方法には，HPLC-ECD（電気化学検出器付きHPLC）法，GC-MS（ガスクロマトグラフィ／質量分析）法，LC-MS/MS（液体クロマトグラフ-タンデム質量分析）法，ELISA法等があり，それぞれ精度，感度，経済性等に長所・短所がある。

3. 7 その他

不定期DNA合成試験，HPRT（hypoxanthine-guanine phosphoribosyltransferase）前進突然変異法，γ-H2AX染色法などがある。不定期DNA合成試験は，主にラットの肝細胞が用いられ，DNA損傷を修復合成するために取り込まれる^{3}H-チミジンを観察する。HPRT前進突然変異法は，ほ乳類雄性細胞（XY染色体）を用いて，同遺伝子の *in vitro* および *in vivo* での変異を評価する。同遺伝子はX染色体上にある。その欠損変異は，6-チオグアニン抵抗性を標識として容易に選別できる。γ-H2AX染色法は，2本鎖DNA切断が生じた場合の最も早期の細胞応答の一つが核内のDNAが巻き付いているヒストンたんぱく質H2AXの139番セリン部位のリン酸化を評価する。γ-H2AX（リン酸化H2AX）に特異的な蛍光標識抗体を用いることで，細胞核内に生じるγ-H2AXを顕微鏡下で観察する。

3. 8 ナノ粒子の遺伝毒性の評価

化学物質の遺伝毒性評価と同じように，単一の遺伝毒性試験で，遺伝子突然変異，染色体異常等全ての遺伝的変化を検出することは出来ない。現在，化学物質の遺伝毒性は，数種の遺伝毒性試験を組み合わせて評価される。遺伝毒性にかかわる法規・ガイドラインにより，Ames試験，染色体異常試験，小核試験等は，組み合わせ・利用法が異なる。例えば，年間100kg以上産生される新規化学物質に対して，労働安全衛生法では，Ames試験を必要項目とし，染色体異常試験はAmes試験陽性，かつ比活性1000以上で追加される。また，医薬品では，薬事法により，Ames試験，染色体異常試験，小核試験を標準的組み合わせとして総合評価する。OECDガイドラインでは，新規化学物質，医療用具に対して，Ames試験，染色体異常試験を標準として，小核試験は必要に応じて追加とされている。この評価と同様にナノ粒子も評価されていくと考えられる。

3. 9 遺伝毒性のメカニズム

吸入，皮膚，口腔粘膜等から入り込んだナノ粒子が各種細胞に様々な機構で取り込まれる。その結果，核内に侵入したナノ粒子が直接DNAあるいはDNAに関連するタンパク質等に物理的損傷を与える場合が考えられる。酸化チタンやシリカのナノ粒子が核内に入り込み，核内タンパク質を凝集させ，DNA複製，転写，細胞増殖等に影響を与えていることが報告されている[7,8]。また，細胞分裂あるいは細胞内のホメオスタシス維持にかかわるタンパク質等に影響を与え，間接的にDNAに損傷を与える場合や，酸化ストレス，炎症，細胞内異常なシグナリングなどを引き起こし，その結果としてDNA障害を与える場合が考えられる。酸化ストレスは，生体における活性酸素種（Reactive Oxygen Species：ROS）と抗酸化システムのバランスであるが，遺伝毒性を引き起こす機構の中で重要な役割を果たすと考えられている。ROSは生体内において，DNA，脂質，タンパク質，酵素等の生体高分子と反応し，生体のホメオスタシスを乱す。ROSに誘導されるDNA損傷は，1本鎖DNA切断，2本鎖DNA切断，8-OHdG付加体形成，DNAクロスリンクがあり，発癌へ結びついている。

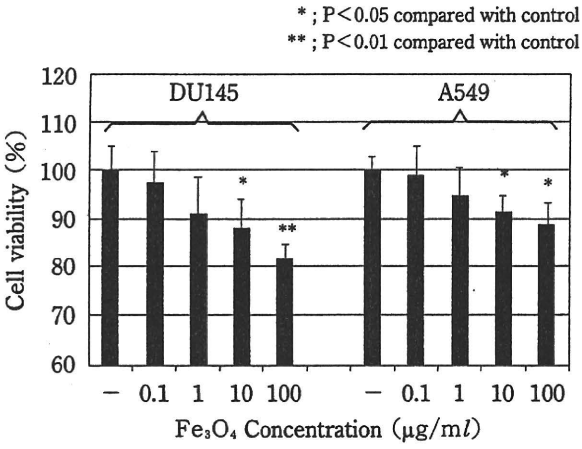

Fig. 3 Alamar Blue assay

4．細胞依存性について

当研究室では，平成20年度より各種ナノ粒子等（マグネタイト Fe_3O_4，二酸化チタン TiO_2，フラーレン C_{60}，カーボンブラック Carbon black，カオリン Kaolin 等）のヒト細胞株（肺癌細胞株 A549，前立腺癌細胞株 LNCaP，DU-145，PC-3 等）への細胞毒性，および遺伝毒性の評価を行っている。細胞毒性評価として，透過型電子顕微鏡によるナノ粒子の細胞内局在，Cytology（細胞形態観察および免疫細胞化学的染色），Alamar blue assay, LDH assay（損傷した細胞膜から漏出した乳酸脱水素酵素を測定），H_2DCF-DA 検出を行い，遺伝毒性評価として，8-OHdG 測定，Comet assay，小核試験等を行っている。共同研究先では，*in vivo* 試験として *gpt*（*guanine phosphoribosyltransferase*）delta transgenic mice にナノ粒子を投与して，*gpt* 変異の解析を行っている[11]。ここで，マグネタイト Fe_3O_4（以下 MNP）の異なる細胞株（A549 および DU-145 細胞株）への影響に関する実験結果の一部を紹介する。使用した MNP の 1 次粒子径は 10 nm で，MNP の 2 次粒子径（培養液中）は 180 nm 程度であった。培養液中では，ナノ粒子は凝集するもナノサイズが維持された状態で，細胞に曝露されていると考えられた。両細胞株とも Cell Viability は，濃度依存的に低下した（**Fig. 3**）。H_2DCF-DA を用いた細胞内の ROS 産生が，前立腺癌細胞株 DU-145，PC-3，LNCaP で確認された。Cytology では，非曝露群の細胞は個々の細胞が vivid で，分裂像も多数認められたが，曝露群の細胞は細胞質の変性，接着性の低下等が認められた。細胞増殖関連抗原 Ki-67 に対する抗体を用いた免疫細胞化学的染色でも，曝露群では Ki-67 陽性細胞の比率が低下し，増殖能の低下が認められた。これらの結果から，

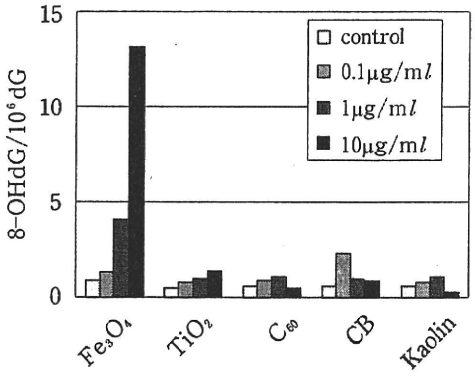

Fig. 4 8-OHdG levels in DNA from DU-145 cells

MNP はこれら細胞株に対して，細胞毒性があると考えられた。一方，MNP 曝露群・非曝露群の 8-OH-dG を測定したところ，DU-145 をはじめとする前立腺癌細胞株で 8-OH-dG 量の増加を認めた（**Fig. 4**）。これは，他のナノ粒子を曝露した時に比べて有意に高値であった。また，A549 肺癌細胞株では，他のナノ粒子と同様に有意な増加は認められなかった（**Fig. 5**）。Comet assay では，陽性コントロールとして使用した青石綿と同程度の DNA 損傷を認めている。これらの結果は，MNP が前立腺癌細胞に特異的に DNA 損傷を与えていると考えられた。MNP は前立腺癌細胞に特異的に細胞毒性および遺伝毒性を与えると推測される。臨床癌も含めて前立腺癌細胞株は，塩基除去修復遺伝子発現の欠損や抗酸化酵素の発現が変化していることが報告されている[12, 13]。MNP が前立腺癌細胞株に ROS を産生させ，塩基除去修復能や抗酸化能が落ちるために，細胞毒性および遺伝毒性を引き起こすと考えられた。MNP 自体は既に生物・医学領域に広く応用展開されている。MNP 自体の毒性評価に関して，発表論文も少なく，比較的安全と考えられていたが，現在，毒性に関する論文が集積し始めている。MNP 自体は，いわゆる Fenton 反応（$H_2O_2+Fe^{2+}\rightarrow Fe^{3+}+HO^-+HO^*$）を介して，ROS を産生すると考えられ

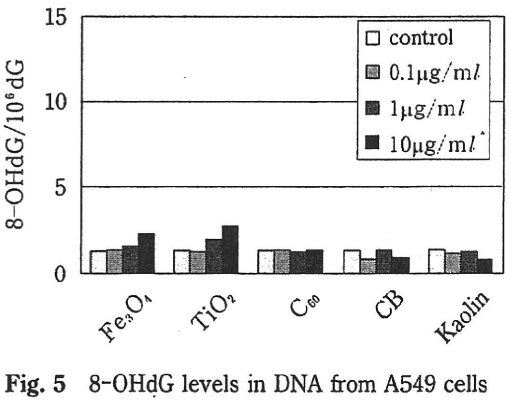

Fig. 5 8-OHdG levels in DNA from A549 cells

ている。必ずしもFenton反応のみではないが，ROSが産生され，酸化ストレスを生じ毒性を引き起こすと考えられている[14]。酸化ストレスの癌細胞への影響は複雑である。多段階発癌の過程で，酸化ストレスは発癌にかかわり，発癌後も癌細胞を悪性度の増す方向へ誘導する[15]。しかし，高度な酸化ストレスでは，逆に癌細胞は死滅し，癌治療にも利用されている。MNPを標的癌細胞に送り込むことにより，癌治療に相乗あるいは相加効果を与える可能性がある。われわれグループは前立腺癌を標的としているが，既にK562/A02白血病細胞を標的にした治療法の報告がされている[16]。

5．結　言

現在まで，ナノ粒子の毒性評価のために，多くの*in vitro*試験の論文が出ているが，細胞株のみならず，培養液，あるいはassay系への影響等と基本的な部分で注意しなければならないことが多い。細胞生物学的な機構の解明も視野にいれて，注意深く細胞毒性および遺伝毒性の解析を行ってゆきたい。

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Low-dose carcinogenicity of 2-amino-3-methylimidazo[4,5-*f*]quinoline in rats: Evidence for the existence of no-effect levels and a mechanism involving p21$^{Cip/WAF1}$

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The carcinogenicity of the low amounts of genotoxic carcinogens present in food is of pressing concern. The purpose of the present study was to determine the carcinogenicity of low doses of the dietary genotoxic carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and to investigate mechanisms by which IQ exerts its carcinogenic effects. A total of 1595 male F344 rats were divided into seven groups and administered with IQ at doses of 0, 0.001, 0.01, 0.1, 1, 10 and 100 p.p.m. in the diet for 16 weeks. We found that IQ doses of 1 p.p.m. and below did not induce preneoplastic lesions in either the liver or the colon, while IQ doses of 10 and 100 p.p.m. induced preneoplastic lesions in both of these organs. These results demonstrate the presence of no-effect levels of IQ for both liver and colon carcinogenicity in rats. The finding that p21$^{Cip/WAF1}$ was significantly induced in the liver at doses well below those required for IQ mediated carcinogenic effects suggests that induction of p21$^{Cip/WAF1}$ is one of the mechanisms responsible for the observed no-effect of low doses of IQ. Furthermore, IQ administration caused significant induction of CYP1A2 at doses of 0.01–10 p.p.m., but administration of 100 p.p.m. IQ induced CYP1A1 rather than CYP1A2. This result indicates the importance of dosage when interpreting data on the carcinogenicity and metabolic activation of IQ. Overall, our results suggest the existence of no-effect levels for the carcinogenicity of this genotoxic compound. (*Cancer Sci* 2011; 102: 88–94)

Exposure to environmental carcinogens is one of the most significant causes of human cancers. Determination of the dose-response relationship between carcinogen exposure and induction of cancer is one of the most important areas of chemical risk assessment. Of particularly high priority is the cancer risk assessment of dietary carcinogens.

Heterocyclic amines (HCA) are well known dietary genotoxic carcinogens derived from cooked protein-rich foods such as meat and fish,[1–3] and the carcinogenicities of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) have been widely investigated in various animal models. MeIQx induces cancers of the liver, zymbal gland, skin and clitoral gland in rats,[4] and caners of the liver and lung, and lymphoma and leukemia in mice.[5] PhIP induces colon cancers and mammary gland cancers in rats,[6] and lymphomas in mice.[7] IQ induces cancers of the liver, colon, mammary and zymbal glands in rats, caners of the liver, lung and forestomach in mice, and cancer of the liver in non-human primates.[8–10] MeIQx and PhIP are classified as category 2B compounds (possibly carcinogenic to humans) and IQ is classified as a category 2A compound (probably carcinogenic to humans) by the International Agency for Research on Cancer.[11] Therefore, although the concentrations of HCA in food are low, they constitute a potential hazard, and there is concern regarding the carcinogenic effects of low doses of these HCA.

Based on the view that even minute doses of a genotoxic carcinogen has the potential to produce irreversible deleterious genetic changes in the DNA of a target organ cell and the argument that if sufficient numbers of test animals are used the carcinogenic effect of a minute dose can be demonstrated, it is generally assumed that genotoxic carcinogens exert a non-threshold carcinogenic effect. However, the carcinogenicities of most genotoxic carcinogens are determined by experimental animal carcinogenicity studies using doses that are generally orders of magnitude higher than actual human exposure levels and the dose-response curves obtained are then extrapolated to zero using a non-threshold mathematical model. This approach, however, is being challenged as advancements in the understanding of the molecular mechanisms of carcinogenesis are being made and experimental evidence showing that genotoxic carcinogens do not exert mutagenic and carcinogenic effects at low doses accumulates.[12–19]

Previously, we demonstrated the existence of no-effect levels of MeIQx for both hepatocarcinogenicity and *in vivo* mutagenicity in various carcinogenesis models in different rat strains.[17,20–22] It has also been shown that low doses of PhIP do not exert either initiation or promotion activities in colon carcinogenesis in the rat.[23,24] However, little is known about the carcinogenic potential of low doses of IQ.

In addition, little is known about the mechanisms underlying the carcinogenicities of lower doses of HCA, but incorporation of mechanistic information is critical for quantitative cancer risk assessment. The purpose of the present study is to determine the relationship between administration of low doses of IQ and induction of preneoplastic lesions in the liver and colon in rats, and to investigate carcinogenic mechanisms of action of various doses of IQ by evaluating DNA-adduct formation, oxidative DNA damage and expression levels of genes involved in metabolic activation of IQ, cell proliferation and DNA damage repair in the liver.

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Materials and Methods

Chemical and diets. IQ was purchased from Nard Institute Ltd (Osaka, Japan) with a purity of 99.9%. Basal diets (powdered MF; Oriental Yeast Co., Tokyo, Japan) and the diets containing IQ were prepared once a month by Oriental Yeast Co.

Animals. A total of 1595 male F344 rats were supplied by Charles River Japan, Inc. (Hino, Shiga, Japan) and were used at 21 days of age. Animals were housed in polycarbonate cages (five per cage) in experimental animal rooms with a targeted temperature of 22 ± 3°C, relative humidity of 55 ± 5% and a 12-h light/dark cycle. Diet and tap water were available *ad libitum* throughout the study.

Experimental design. The animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School. Rats were randomized into seven groups, 245 rats in each of groups 1–6 and 125 rats in group 7. Since the levels of IQ in cooked foods are lower than those of MeIQx and PhIP,[11] IQ dosage and treatment duration in this study were the same as the previous low dose carcinogenicity studies with MeIQx and PhIP.[18,24] Animals were fed diets containing IQ as follows: 0 (group 1, control), 0.001 (group 2), 0.01 (group 3), 0.1 (group 4), 1 (group 5), 10 (group 6) and 100 p.p.m. (group 7) for 16 weeks. Fresh diet was supplied to the animals twice weekly. Bodyweights, food consumption and water intake were measured weekly.

Five rats in each group were killed at week 4 under ether anesthesia. At death, livers were snap frozen in liquid nitrogen and stored at −80°C for examination of IQ-DNA adducts and 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation in the DNA. The remaining rats were killed at the end of week 16 under ether anesthesia for examination of the development of glutathione *S*-transferase placental form (GST-P) positive foci, which is a well-established preneoplastic lesion in the rat liver,[25,26] and aberrant crypt foci (ACF), which is a surrogate marker for preneoplastic lesions in the rat colon.[24,27,28] At death, livers were excised, weighed and then three slices each from the left lateral, medial and right lateral lobes were cut and placed in 10% phosphate-buffered formalin. The remaining liver tissues were snap frozen in liquid nitrogen and stored at −80°C for mRNA expression analysis. Following fixation, liver tissues were embedded in paraffin and processed for histopathological examination.

Examination of GST-P positive foci in the liver. Anti-rat GST-P polyclonal antibody (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan) at a dilution of 1:1000 was used for immunohistochemical staining of GST-P. The GST-P-positive hepatocellular foci composed of two or more cells were counted under a light microscope.[17,18,20,22] Total areas of livers were measured using a color image processor IPAP (Sumica Technos, Osaka, Japan) and the number of GST-P-positive foci per square centimeter of liver tissue was calculated.

IQ-DNA adduct and 8-OHdG formation in livers. IQ-DNA adducts were measured by the ^{32}P-postlabeling method as described previously.[29,30] Levels of 8-OHdG formation in liver DNA were determined by high-performance liquid chromatography with electrochemical detection as previously described.[31]

TaqMan real-time quantitative PCR. The mRNA expression levels of genes involved in IQ metabolism (CYP1A1, CYP1A2 and CYP1B1), DNA damage repair (8-oxoguanine DNA glycosylase [Ogg1], growth arrest and DNA damage-inducible protein 45 [GADD45], AP endonuclease-1 [APE-1], MSH2 and MSH3) and cell cycle regulation (p53 and $p21^{Cip/WAF1}$ and proliferating cell nuclear antigen [PCNA]) were evaluated in the livers by TaqMan real-time quantitative PCR as described previously.[31] Sequence-specific primers and probes (Taqman Gene Expression Assay) were purchased from Applied Biosystems, Inc., Carlsbad, CA, USA. Beta-2-microglobulin (B2M) was used as an internal control.

Examination of ACF in colon. Formation of ACF was examined as described previously.[24] Although ACF consisting of four or more crypts are considered to be better predictors of colon tumor outcome in rats,[32] to ensure that all doses of IQ that have the potential to induce colon carcinogenesis were accounted for, doses of IQ that caused an increase of any size of ACF were considered to have the potential to induce colon carcinogenesis in the present study.[24]

Statistical analysis. All mean values are reported as mean ± SD. Statistical analyses were performed using the Statlight program (Yukms Co., Ltd, Tokyo, Japan). Homogeneity of variance was tested by the Bartlett test. Differences in mean values between the control and IQ-treated groups were evaluated by the 2-tailed Dunnett test when variance was homogeneous and the 2-tailed Steel test when variance was heterogeneous.[22,31] P values <0.05 were considered significant.

Results

General observation. All animals survived to the end of study without any apparent abnormal pathological features. The final average body and liver weights and IQ intake are summarized in Table 1. The final bodyweight of the 100 p.p.m. group was significantly lower than that of the 0 p.p.m. group. Absolute and relative liver weights were significantly decreased in the 0.1 and 1 p.p.m. groups and were significantly increased in the 100 p.p.m. group compared with the 0 p.p.m. group. There were no significant differences in either food or water consumption among groups (data not shown). The intake of IQ was proportional to the administered doses (Table 1). No tumors were found in any organs including the liver and colon in any of the groups.

Induction of GST-P-positive foci in the livers. No histopathological changes were observed in any of the IQ-treated groups.

Table 1. Body and organ weights, and IQ intake

Group	IQ (p.p.m.)	No. rats	Bodyweight (g)	Liver		Average IQ intake	
				Absolute weight (g)	Relative weight (%)	Daily intake (mg/kg b.w.)	Total (mg/kg b.w.)
1	0	240	331 ± 23	9.3 ± 1.7	2.8 ± 0.4	0	
2	0.001	240	332 ± 17	9.1 ± 1.4	2.8 ± 0.4	0.0001	0.008
3	0.01	240	331 ± 19	9.0 ± 1.5	2.8 ± 0.4	0.0007	0.08
4	0.1	240	331 ± 22	8.5 ± 1.2*	2.6 ± 0.3*	0.008	0.9
5	1	240	331 ± 17	8.5 ± 1.2*	2.6 ± 0.3*	0.08	8.7
6	10	240	330 ± 18	9.0 ± 1.3	2.7 ± 0.4	0.76	85.1
7	100	120	319 ± 19*	10.0 ± 1.6*	3.2 ± 0.4*	7.83	877.5

*Significantly different from group 1. IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.