Table 1. Yield of m^5dC from dC by BuOOH/Fe^{2+} Treatment[a]

reaction condition		yield of $m^5dC/10^4dC$
1 N H_2SO_4		25.4
0.35 N H_2SO_4		21.3
pH 4.5	air	6.01
	N_2	5.94
pH 7.4	air	1.76
	N_2	2.19

[a] dC (final concentration, 5.46 mM) and $FeSO_4$ (4 mM) were mixed in 0.32 mL of 20 mM sodium acetate buffer (pH 4.5), 20 mM phosphate buffer (pH 7.4), or in H_2SO_4 solutions (0.35 or 1 N), and the reaction was started by adding BuOOH (final concentration, 16 mM) in a sealed plastic tube (tube volume, 2 mL) by vigorous shaking at 20 °C. In some experiments, the oxygen in the solution was removed by flushing with nitrogen gas before starting the reactions. After a 30 min reaction, the solution was centrifuged, and an aliquot of the supernatant was injected into the HPLC apparatus. For the reactions in H_2SO_4 solutions, the supernatant was neutralized with 5 M NaOH, and then, an aliquot was injected into the HPLC apparatus.

the CuOOH concentration with the same concentration of Fe^{2+}. When a radical scavenger, TEMPO or POBN, was added to the reaction mixture at a concentration up to 3 mM, the m^5dC formation was inhibited (Figure 3). It should be mentioned that POBN is an efficient trapping agent for methyl radicals (*8*), while TEMPO, in addition to combining with methyl radicals, oxidizes Fe^{2+}, which is an important factor to produce methyl radicals (*13*). From these results, it can be concluded that this reaction proceeds via a free radical mechanism, probably via a methyl radical.

Reaction of dC with BuOOH/Fe^{2+}. The formation of m^5dC from dC was also observed after a reaction with BuOOH/Fe^{2+} at pH 7.4 (Table 1). Acidification of the reaction conditions increased the yield of m^5dC. When oxygen was removed from the reaction mixture at pH 7.4, the yield increased by 25%. The methyl radicals produced in the reaction may partly react with oxygen under aerobic conditions to form methyl peroxy radicals and then further decompose to formaldehyde.

Reaction of DNA Polymers with CuOOH/Fe^{2+}. After a double-stranded homopolymer, poly(dG)•poly(dC), or an alternating copolymer, poly(dG-dC)•poly(dG-dC), was reacted with CuOOH in the presence of Fe^{2+} at pH 7.4, the formation of m^5dC was examined by an immunodot blot analysis. The formation of m^5dC was clearly detected in both DNA polymers after the treatment (Figure 4). As a positive control, we analyzed 0.1, 1, and 10 ng of calf thymus DNA, because it contains 1.39 mol % m^5dC. The chemiluminescence intensity increased depending upon the calf thymus DNA concentration. The control DNA polymers without treatment also showed weak chemiluminescence. This means that commercial DNA polymers contain a small amount of m^5dC. We considered the immunodot blot analysis to be semiquantitative; therefore, the exact amount of m^5dC in the reaction mixture was analyzed by the LC/MS/MS method.

Confirmation of m^5dC Formation in the Reaction Mixtures by LC/MS/MS Analysis. In the LC/MS analysis, the standard m^5dC exhibited an MH^+ ion at *m/z* 242, and product ion analysis from *m/z* 242 with 11 eV revealed a fragment BH_2^+ ion at *m/z* 126 that is formed by the loss of 2′-deoxyribose (Figure 5). Therefore, the m^5dC in the reaction mixture was analyzed by LC/MS/MS, by monitoring the *m/z* 242 → 126 transition. In Figure 6, chromatograms of the LC/MS/MS analysis of standard m^5dC (A), dC-BuOOH/ Fe^{2+}(B), and DNA polymer- CuOOH/ Fe^{2+}(C) are shown. In both the dC- and the polymer DNA-product analysis, a 242 → 126 transition peak

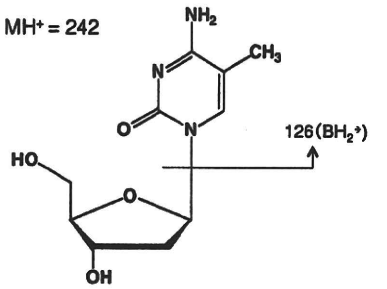

Figure 5. MH^+ ion of m^5dC and the product ion BH_2^+ in the LC/MS/MS analysis.

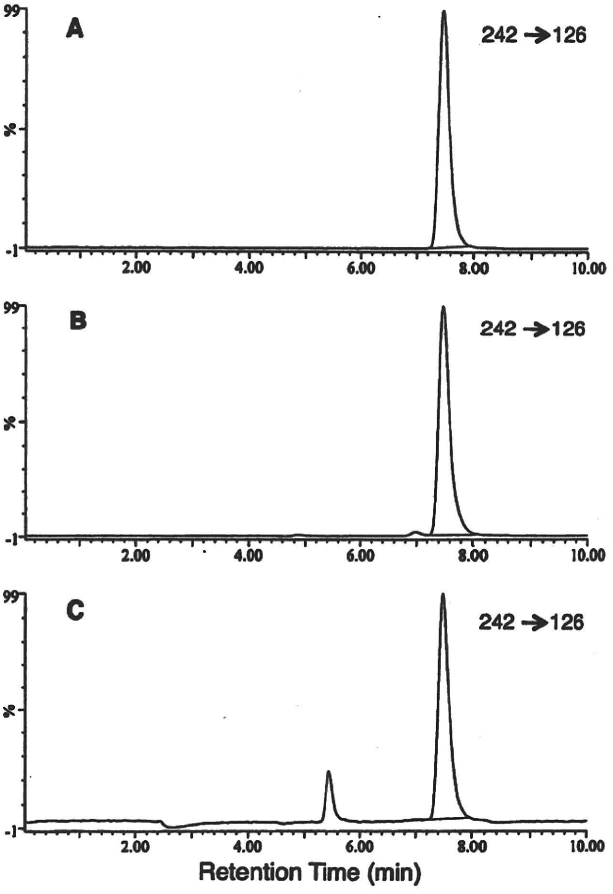

Figure 6. LC/MS/MS analysis of the reaction products. (A) Standard m^5dC (356 ng/mL), (B) dC-BuOOH/Fe^{2+} reaction mixture (reaction conditions were the same as in Table 1, at pH 7.4 and with air), and (C) hydrolysate of poly(dG-dC)•poly(dG-dC)-CuOOH/Fe^{2+} reaction product (reaction conditions were the same as in Figure 4). A 2 μL portion of each sample was injected. The transition *m/z* 242 → 126 was monitored.

appeared at 7.47 min, which is the same retention time as that of authentic m^5dC.

On the basis of the LC/MS/MS analysis, the yield of m^5dC in the dC-BuOOH/Fe^{2+} reaction was calculated to be $1.97/10^4$ dC, which is comparable to that estimated by HPLC-UV ($1.76/10^4$ dC, see Table 1). On the other hand, the yield of m^5dC in the DNA polymer/CuOOH/Fe^{2+} reaction was $2.97/10^5$ dC, while that from dC with the same reaction condition was $2.85/10^4$ dC (see Figure 2B). Therefore, the yield of m^5dC formation is 10-fold lower in DNA than in the dC monomer.

Discussion

A generally accepted concept of the mechanism of de novo DNA methylation during carcinogenesis is the enzymatic

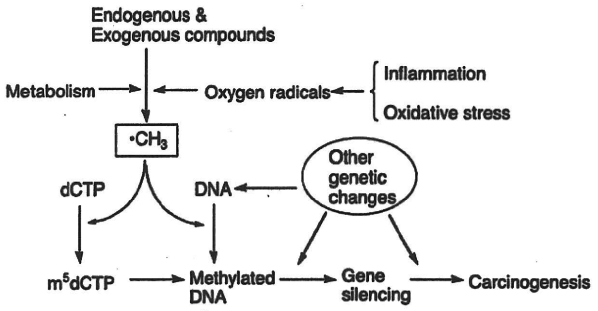

Figure 7. Hypothetical formation of m^5dC in nucleotides and DNA via methyl radicals.

reaction by DNMT3b, using *S*-adenosylmethionine as the methyl donor. Correlations between DNMT3b polymorphisms and neoplastic outcomes have been reported (*14, 15*). It was also reported that tumor suppressor gene inactivation during cadmium-induced transformation is correlated with the overexpression of the de novo DNA methyltransferase DNMT3b (*16*). In the present study, a free radical mechanism to produce m^5dC in DNA or the nucleotide pool was proposed, because the C-5 position of cytosine is an active site for free radical reactions. We observed the methylation of the C-5 position of cytosine in the nucleoside and DNA by the tumor promoters, BuOOH and CuOOH, in the presence of Fe^{2+} via a free radical mechanism. The generation of methyl radicals from organic hydroperoxide tumor promoters in vitro and in isolated mouse keratinocytes has been previously characterized by ESR (*10*). In addition to these chemicals, the generation of methyl radicals by the chemical and biological transformation of various carcinogens has been characterized in vitro and in vivo. For instance, methyl hydrazine derivatives, such as 1,2-dimethylhydrazine (*17*) and procarbazine (*18*), are metabolized to methyl radicals. Acetaldehyde, which is an important human carcinogen related to smoking, drinking, and inflammation (*19, 20*), generates methyl radicals upon treatment with xanthine oxidase (*21*), peroxynitrite (*22*), and iron (II)/hydrogen peroxide (*22*). The amino acid methionine produces a methyl radical upon γ-irradiation (*23*) and by the treatment of its sulfoxide derivative with peroxynitrite (*24*).

During tumor promotion in mouse skin by cigarette smoke condensate, hypermethylation in the promoter regions of the HoxA5, p16, and MGMT genes and their inactivation are important mechanisms of clonal expansion (*25*). Cigarette smoke is known to generate oxygen and carbon radicals (*26*). CuOOH treatment also reportedly induces malignant carcinomas in DMBA, TPA-carcinogenesis experiments with mice (*27, 28*). Therefore, DNA hypermethylation is an important mechanism of tumor promotion and progression.

Our results indicate that the formation of m^5dC in DNA does not seem to be specific to the CpG sequence, and the yield is rather low. Even if the methylation is a rare reaction, the m^5dC thus produced in DNA is not repaired, and its formation in CpG sequences would accumulate during continuous cell divisions by the maintenance DNA methyltransferase, DNMT1. When these modifications occur by chance in the promoter sequences of tumor suppressor genes, these cells will acquire a growth advantage over the surrounding cells, which will be further accelerated with other genetic changes.

It is interesting to speculate that m^5dCTP is formed from dCTP by methyl radicals in the nucleotide pool and then is incorporated into DNA (Figure 7), because we found that the yield of m^5dC as the monomer is much higher than that in DNA in the present study. It has been reported that m^5dCTP introduced into cultured CHO V-79 cells by electroporation is incorporated into DNA and induces gene silencing (*29*).

In conclusion, we found the methyl radical mediated formation of m^5dC from dC or in DNA, by a treatment with CuOOH or BuOOH in the presence of ferrous ion at pH 7.4. We have extended our finding to the following hypothesis. Methyl radicals are produced from the metabolism of carcinogens or from endogenous compounds attacked by oxidants generated by inflammation, ionizing radiation, and other oxidative stresses, and they modify dCTP and DNA to form m^5dC (Figure 7). The accumulation of this chemical modification may be one of the mechanisms of epigenetic change to induce gene silencing and carcinogenesis.

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Effect of age, smoking and other lifestyle factors on urinary 7-methylguanine and 8-hydroxydeoxyguanosine

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Urinary 8-hydroxydeoxyguanosine (8-OH-dG) and 7-methylguanine (m^7Gua) were measured by a column-switching high performance liquid chromatography method as markers of oxidative and methylating DNA damage, respectively. We investigated the associations between urinary 8-OH-dG or m^7Gua and various lifestyle and demographic factors, such as age and sex. The urinary 8-OH-dG excretion level was positively correlated with cigarette smoking, but inversely correlated with fruit consumption, physical activity and total energy consumed per day. A multiple regression analysis revealed that daily physical activity and healthy meal combinations decreased the urinary 8-OH-dG level, whereas alcohol consumption increased it. In terms of the urinary m^7Gua measurement, cigarette smoking, age and consumption of meat, fish, egg, soybean, etc. were positively correlated with the urinary m^7Gua level, whereas body weight, BMI, physical activity, and dietary index score, which indicates good nutritional balance, were negatively correlated with the amount of m^7Gua. Based on a multiple regression analysis, cigarette smoking and age correlated with the m^7Gua level, while high BMI and healthy meal combinations have significant reducing effects on m^7Gua level. Therefore, the urinary m^7Gua level is considered to be a useful marker of DNA methylation, not only from smoking, but also from aging and unhealthy dietary habits. (*Cancer Sci* 2009; 100: 715–721)

Oxygen radicals are formed in cells by oxygen metabolism and various environmental agents, and they damage DNA, RNA, and proteins.[1] Among the many types of oxidative DNA damage, 8-hydroxydeoxyguanosine (8-OH-dG) is a major product and is frequently analyzed as a marker of cellular oxidative stress related to carcinogenesis,[2,3] because 8-OH-dG induces mutations,[4,5] is excreted in the urine, and it has been analyzed by high performance liquid chromatography-electrochemical detection (HPLC-ECD),[6,7] liquid chromatography-tandem mass spectrometry (LC-MS),[8] gas chromatography-mass spectrometry (GC-MS),[9] and enzyme linked immunosorbent assay (ELISA).[10] However, the reproducibility and accuracy of its measurement are much higher with the HPLC-ECD and LC-MS/MS methods, as compared to the ELISA method.[11,12] We have reported that higher 8-OH-dG levels were observed in the lung DNA of smokers,[13] the liver DNA of chronic hepatitis patients,[14] and in the stomach DNA of patients infected with *Helicobacter pylori*.[15] It has also been reported that the urinary 8-OH-dG level is higher in cancer patients than in healthy people,[16] higher in smokers than in non-smokers,[17] and lower in people who exercise moderately.[17] In addition, the urinary 8-OH-dG level was higher in men than in women,[7] and it negatively correlated to body mass index (BMI).[7] As an explanation for the relationship between a lean BMI and high urinary 8-OH-dG excretion, it has been suggested that lean persons have a higher metabolic rate than obese persons,[18] and therefore have higher oxidative stress. Thus, various factors affect the 8-OH-dG levels in humans.

On the other hand, 7-methylguanine (m^7Gua) is a biomarker of DNA damage induced by methylating agents. m^7Gua may serve as a good biomarker of DNA damage caused by nitrosamines in tobacco smoke,[19] and other environmental methylating agents, such as methyl bromide.[20] It is also possible that m^7Gua is formed in cellular DNA by an endogenous methylating agent, S-adenosylmethionine.[21] m^7Gua is also a degradation product from RNA,[22,23] and is known as a metabolic rate marker. Urinary m^7Gua was measured by several researchers,[24] as a product of DNA damage. For instance, the amount of m^7Gua excreted in the urine is increased after exposure to methylating agents in laboratory animals.[25,26] Higher levels of m^7Gua excretion have been reported among patients with colon cancer,[27] although not in patients with gastric cancer.[28] In particular, the urinary excretion of m^7Gua has been shown to be higher among smokers than non-smokers.[29]

Therefore, urinary 8-OH-dG and m^7Gua seem to be useful biomarkers of DNA damage caused by oxidation and methylation, respectively. Measuring the two markers may be very meaningful, because the mechanisms of mutagenesis and carcinogenesis due to DNA oxidation and methylation are different. Therefore, it would be beneficial if the amounts of 8-OH-dG and m^7Gua in human urine could be analyzed simultaneously. Recently, we developed a new HPLC method to analyze 8-OH-dG and m^7Gua simultaneously, based on an anion exchange and reverse phase column-switching system.[30] This HPLC method was further modified to measure 8-OH-dG and m^7Gua in not only human urine samples, but also those from rat and mouse.[30] In this study, with this new HPLC method, we examined the influence of various lifestyle factors on the levels of urinary 8-OH-dG and urinary m^7Gua among a sample of 361 Japanese healthy male employees.

Materials and Methods

Urine collection and questionnaire investigation. After informed consent was obtained, urine samples were collected from 578 healthy employees in a steel-manufacturing company. At the same time, each individual's information on age, height and weight (for BMI), sex, status of cigarette smoking and alcohol drinking, status of dietary habits (for dietary score), status of rest (for rest score), and status of daily physical activity was obtained through a questionnaire. However, in the present study

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Table 1. The characteristics of categorical lifestyle factors and urinary 8-hydroxydeoxyguanosine (8-OH-dG) levels and urinary 7-methylguanine (m^7Gua) levels in 361 male subjects

Variables	Category	*n*	%	Urinary markers†			
				8-OH-dG	*P**	m^7Gua	*P**
Sleep	Deficient	10	2.8	4.37 ± 0.25	0.53	8.03 ± 0.47	0.26
	Slightly deficient	181	50.1	4.19 ± 0.12		8.98 ± 0.21	
	Sufficient	170	47.1	4.20 ± 0.11		8.60 ± 0.19	
Holiday	Little or none	2	0.6	5.41 ± 1.20	1.00	9.49 ± 0.12	0.15
	Once a week	51	14.1	3.96 ± 0.21		9.42 ± 0.40	
	Twice a week	308	85.3	4.23 ± 0.08		8.66 ± 0.15	
Fatigue	Always	18	5.0	3.66 ± 0.32	0.86	8.07 ± 0.36	0.37
	Sometimes	256	70.9	4.17 ± 0.09		8.87 ± 0.17	
	Rarely	87	24.1	4.39 ± 0.17		8.62 ± 0.25	
Rhythm	Irregular	66	18.3	4.05 ± 0.19	0.25	8.28 ± 0.27	0.24
	Mostly regular	215	59.6	4.20 ± 0.10		8.87 ± 0.19	
	Regular	80	22.2	4.30 ± 0.16		8.91 ± 0.28	
Refreshing	Difficult	13	3.6	4.40 ± 0.38	0.48	7.85 ± 0.56	0.36
	Moderate	255	70.6	4.13 ± 0.09		8.86 ± 0.17	
	Easy	93	25.8	4.33 ± 0.17		8.67 ± 0.23	
Size of a meal	Full stomach every time	24	6.6	3.87 ± 0.27	0.85	8.84 ± 0.55	0.55
	No pattern	198	54.8	4.29 ± 0.11		8.90 ± 0.20	
	Moderation every time	139	38.5	4.12 ± 0.11		8.58 ± 0.20	
Healthy	Rerely	46	12.7	4.25 ± 0.20	0.11	8.57 ± 0.37	0.72
Meal	Consider sometimes	187	51.8	4.33 ± 0.12		8.87 ± 0.20	
Combination	Consider every time	128	35.5	3.99 ± 0.11		8.70 ± 0.23	
Skipping meals	One meal every day	63	17.5	4.37 ± 0.20	0.62	9.32 ± 0.39	0.10
	2 or 3 meals a week	103	28.5	4.09 ± 0.15		8.88 ± 0.28	
	Rarely	195	54.0	4.20 ± 0.10		8.53 ± 0.16	
Light-colored	Rarely	17	4.7	4.32 ± 0.42	0.78	9.30 ± 0.94	0.35
Vegetable	Once a day	268	74.2	4.22 ± 0.09		8.83 ± 0.16	
	Each meal	76	21.1	4.09 ± 0.14		8.43 ± 0.29	
Green- and yellow-collored vegetables	Rarely	26	7.2	4.10 ± 0.32	0.75	9.93 ± 0.68	0.06
	2 or 3 times a week	244	67.6	4.21 ± 0.10		8.74 ± 0.16	
	Everyday	91	25.2	4.18 ± 0.12		8.53 ± 0.25	
Fruit	Rarely	140	38.8	4.24 ± 0.12	*0.03*	8.54 ± 0.22	0.06
	2 or 3 times a week	187	51.8	4.28 ± 0.11		9.07 ± 0.20	
	Everyday	34	9.4	3.57 ± 0.19		8.10 ± 0.36	
Meat, fish, egg, etc.	Rarely	11	3.0	4.72 ± 0.49	0.16	8.15 ± 0.55	*0.05*
	Twice a day	194	53.7	4.25 ± 0.11		9.08 ± 0.19	
	Each meal	156	43.2	4.09 ± 0.11		8.43 ± 0.21	
Milk	Rarely	118	32.7	4.32 ± 0.16	0.23	8.86 ± 0.24	0.68
	2 or 3 times a week	171	47.4	4.10 ± 0.10		8.82 ± 0.21	
	Everyday	72	19.9	4.23 ± 0.15		8.53 ± 0.29	
Oil	Rarely	9	2.5	4.41 ± 0.58	0.55	9.30 ± 1.20	0.26
	2 or 3 times a week	192	53.2	4.15 ± 0.12		8.95 ± 0.19	
	Eeveryday	160	44.3	4.24 ± 0.10		8.52 ± 0.20	
Seaweed	Rarely	58	16.1	3.99 ± 0.18	0.75	8.42 ± 0.34	0.50
	2 or 3 times a week	266	73.7	4.26 ± 0.09		8.86 ± 0.16	
	Everyday	37	10.2	4.04 ± 0.21		8.69 ± 0.49	
Physical	Light	192	53.2	4.39 ± 0.11	*0.03*	8.71 ± 0.17	0.35
activity-1	Moderate	119	33.0	4.09 ± 0.12		9.07 ± 0.28	
	Moderately heavy	12	3.3	3.85 ± 0.48		8.23 ± 0.67	
	Heavy	38	10.5	3.68 ± 0.19		8.32 ± 0.41	

*One-way analysis of variance.
†Data are mean ± SE: 8-OH-dG (μg/g creatinine), m^7Gua (mg/g creatinine).

we only selected the participants who answered all of the items in the questionnaire, to avoid bias as much as possible. Consequently, the data from 361 male subjects (aged 18–59 years, mean 36.3 ± 10.3) were analyzed.

With regard to the questionnaire, the total scores of rest and meals (rest index, dietary index) were expressed as the sum of each score. For example, the rest index score is the sum of the scores (1–3) of sleeping hours, frequency of holidays, feeling of fatigue, rhythm of daily life, and ability to refresh (Table 1). Therefore, a low rest index score means insufficient rest, whereas a high score shows sufficient rest status. Similarly, the status of the dietary habits (dietary index score) is the total score of 10 items consisting of meal size, healthy combinations of meals, frequency of skipping meals, intake of light-colored vegetables, green- and yellow-colored vegetables, fruits, milk, edible oil, seaweed, and intake of meat, fish, egg, soybean, etc. Consequently, a

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Table 2. Association of 8-hydroxydeoxyguanosine (8-OH-dG) and 7-methylguanine (m^7Gua) with continuous variables

Variables	Mean ± SE	Min	Max	Correlation coefficient			
				8-OH-dG	*P*	m^7Gua	*P*
Age	36.28 ± 0.54	18	59	−0.014	0.698	0.190	*<0.001*
Weight	67.14 ± 0.52	45.6	104	−0.033	0.343	−0.094	*0.008*
BMI	22.63 ± 0.16	15.4	34.0	−0.065	0.066	−0.078	*0.028*
Energy consumed	2487.10 ± 21.72	1892	3916	−0.069	*0.049*	−0.086	*0.015*
Physical activity-2	82.92 ± 5.90	3	1207	0.040	0.258	−0.057	0.11
Alcohol drinking	0.89 ± 0.05	0	4.3	0.065	0.073	0.047	0.191
Smoking	15.50 ± 0.57	0	40	0.088	*0.023*	0.247	*<0.001*
Brinkman index	259.79 ± 14.45	0	1520	0.082	*0.024*	0.278	*<0.001*
Rest index score	11.75 ± 0.09	6	15	0.069	0.071	−0.005	0.902
Dietary index score	21.56 ± 0.16	13	30	−0.011	0.757	−0.077	*0.036*

high dietary index score means good nutritional balance. Physical activity was calculated by two different methods (physical activity-1, -2), based on resting metabolic rate (RMR) and physical activity by commuting, working and sports, etc. Namely, 'physical activity-1' was calculated by the ratio of (physical activity by commuting, working and sports/RMR) and was categorized into four groups (scores from 1 to 4). A higher value means high physical activity. 'Physical activity-2' was calculated by physical activity due to commuting and sports, age, sex and weight and was expressed by kCal/day. Total energy consumed per day was calculated from height, age, sex and physical activity by commuting, working and sports, and was expressed by kCal/day. As continuous variables, age, weight, BMI, total energy consumed (KCal/day), physical activity-2 (kCal/day), alcohol drinking (number of glasses drunk per day: converted to Japanese sake), cigarette smoking (number of cigarettes smoked per day) and Brinkman index obtained through the questionnaire were used.

Analysis of m^7Gua, 8-OH-dG and creatinine. Urinary m^7Gua and 8-OH-dG were determined by the method previously described,[31] Briefly, a human urine sample was mixed with the same volume of a dilution solution containing the ribonucleoside marker, 8-hydroxyguanosine. A 20-μL aliquot of the diluted urine sample was injected into HPLC-1 (MCI GEL CA08F, 7 μm, 1.5 × 120 mm; elution, 2% acetonitrile in 0.3 mM sulfuric acid, 50 μL/min, 65°C), via the guard column (1.5 × 40 mm), and the chromatograms were recorded by a Gilson UV detector (UV/VIS-155 with 0.2 mm light path cell). Creatinine and m^7Gua were detected at 245 and 305 nm, respectively. The 8-OH-dG fraction was collected, depending on the relative elution position from the peak of the added marker, 8-OH-G, and was automatically injected into the HPLC-2 column. The 8-OH-dG fraction was fractionated by the HPLC-2 column (Shiseido, Capcell Pak C18, 5 μm, 4.6 × 250 mm; elution, 10 mM sodium phosphate buffer [pH 6.7] containing 5% methanol and an antiseptic Reagent MB [100 μL/L], 1 mL/min, 40°C). The 8-OH-dG was detected by a Coulochem II EC detector (ESA Inc., Chemsford, MA, USA) with a guard cell (5020) and an analytical cell (5011) (applied voltage: guard cell, 350 mV; E1, 170 mV; E2, 300 mV).

Statistics. The relationships between the urinary 8-OH-dG levels and the urinary m^7Gua levels with categorical and continuous variables were analyzed by using oneway analysis of variance (ANOVA) and Kendall's rank correlation coefficients, respectively. In addition to the ANOVA analysis, multiple comparisons between groups were conducted with Scheffe's test. Since the distributions of 8-OH-dG and m^7Gua were skewed, the log-transformed values of 8-OH-dG and m^7Gua, which showed normal distributions, were used in the multiple regression analysis. *P*-values less than 0.05 (two-tailed) were considered to indicate significant differences. All data were analyzed using the SPSS statistical package (SPSS, Chicago, IL, USA) for Windows 14.0.

Results

The mean level of urinary 8-OH-dG (μg/g creatinine) in the 361 male subjects was 4.20 ± 1.47 (SD). A 19.4-fold interindividual variation was found (0.53–10.28 μg/g creatinine). The mean level of m^7Gua normalized to creatinine (mg/g creatinine) was 8.77 ± 2.61 (SD), and a 4.80-fold interindividual variation was found (3.94–18.93 mg/g creatinine). The relationships between the 16 categorical lifestyle factors and the urinary 8-OH-dG level or the urinary m^7Gua level are shown in Table 1. The ANOVA analysis revealed that the urinary 8-OH-dG level was significantly negatively related to fruit consumption ($P = 0.03$) and physical activity-1 ($P = 0.03$). It is noteworthy that the urinary 8-OH-dG levels of the 'rarely' and 'two or three times per week' groups were significantly higher than that of the 'everyday' group ($P = 0.03$) in the fruits item. The results of the Scheffe's test also indicated that fruit consumption significantly reduced the urinary 8-OH-dG level. On the other hand, only the intake of meat, fish, egg, soybean, etc. significantly influenced the m^7Gua excretion ($P < 0.05$). Although the Scheffe's test was conducted to facilitate multiple comparisons of the urinary m^7Gua levels between the groups, no significant differences were observed in all categorical variables.

Table 2 shows the correlations of the continuous variables to the 8-OH-dG (μg/g creatinine) and m^7Gua (mg/g creatinine) levels. Significant positive correlations were observed between the urinary 8-OH-dG level and the average number of cigarettes smoked per day ($r = 0.088$, $P = 0.023$) and the Brinkman index ($r = 0.082$, $P = 0.024$), whereas there were significant inverse correlations between the urinary 8-OH-dG level and physical activity-2 ($r = -0.069$, $P = 0.049$). In contrast, more factors affect the m^7Gua levels. Namely, significant positive correlations were observed between the urinary m^7Gua level and age ($r = 0.190$, $P < 0.001$), the average number of cigarettes smoked per day ($r = 0.247$, $P < 0.001$) and the Brinkman index ($r = 0.278$, $P < 0.001$), whereas significant inverse correlations were observed between the urinary m^7Gua level and weight ($r = -0.094$, $P = 0.008$), BMI ($r = -0.078$, $P = 0.028$), total energy consumed ($r = -0.086$, $P = 0.015$), and the total score obtained from the meal index ($r = -0.077$, $P = 0.036$). In particular, the relationships of age, cigarettes smoked per day and Brinkman index with urinary m^7Gua excretion were remarkable, as shown in Figs 1, 2 and 3, respectively.

The results of the multiple regression analysis of 8-OH-dG by the stepwise method in the 361 male subjects are shown in Table 3. Due to the significant correlation between fatigue and rest score ($r = -0.728$, $P < 0.001$), the fatigue item was not used in the analysis, to avoid collinearity. Similarly, there was a significant correlation between cigarettes smoked per day and Brinkman index ($r = 0.725$, $P < 0.001$), so the Brinkman index item was not included in the analysis. Accordingly, the following

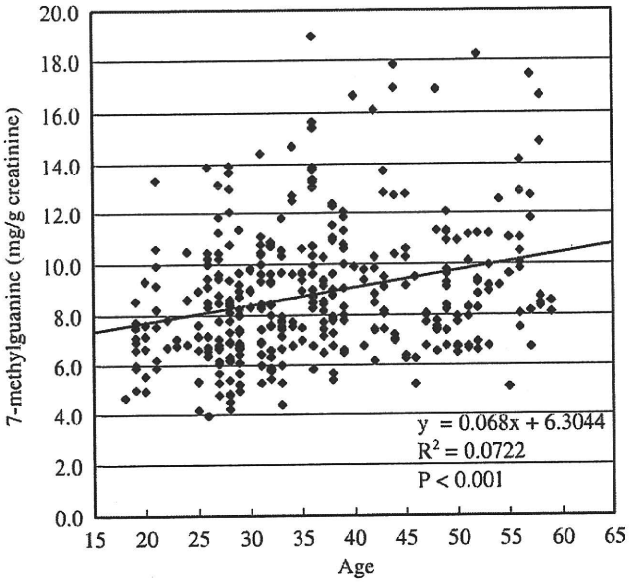

Fig. 1. Association between age and urinary 7-methylguanine level.

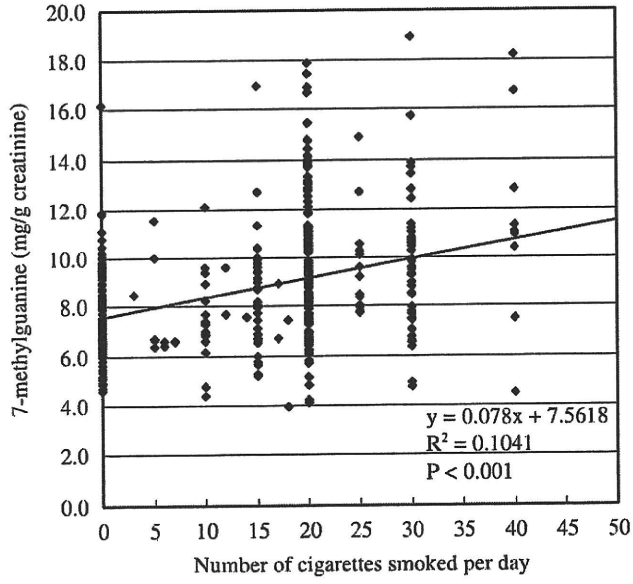

Fig. 2. Association between cigarettes smoked per day and urinary 7-methylguanine level.

24 items were used in the analysis as the independent variables for the subjects with complete data: 15 categorical variables consisting of sleep, holiday, rhythm of daily life, ability to refresh, size of a meal, healthy meal combination, frequency of skipping meals, consumption of light-colored vegetables, green- and yellow-colored vegetables, fruit, meat, milk, oil, seaweed, and physical activity-1, and nine continuous variables consisting of age, weight, BMI, energy consumption, total energy consumed, alcohol drinking, cigarette smoking, rest index score, and dietary index score. The results of the multiple regression analysis by the stepwise method indicated that physical activity-1 and healthy meal combination decreased the urinary 8-OH-dG level, whereas alcohol drinking significantly increased it. The consumption of meat, fish, egg, soybean, etc. showed a tendency to reduce the 8-OH-dG level, and the intakes of green- and yellow-

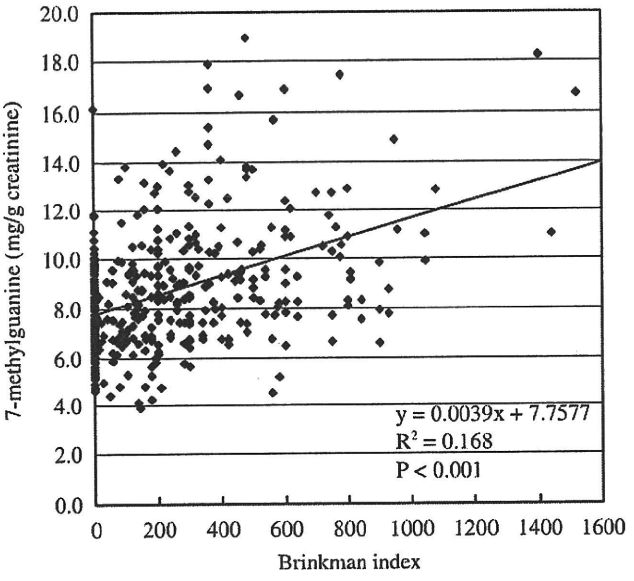

Fig. 3. Association between Brinkman index and the urinary 7-methylguanine level.

colored vegetables showed a tendency of increasing it. These five independent factors obtained from the multiple regression analysis (Table 3) explain only 5.6% of the total variance. On the other hand, the total energy consumed, cigarette smoking, and BMI were not correlated with the urinary 8-OH-dG level.

Table 4 shows the results of the multiple regression analysis using m^7Gua as the dependent variable. The 24 items as above were used in the regression analysis as the independent variables. As a result, cigarette smoking and age were significantly correlated to the urinary m^7Gua level, whereas high BMI and dietary index score (healthy meal style) were negatively correlated to it. These four independent factors obtained from the multiple regression analysis explain 19.6% of the entire variation.

Discussion

In this article, we analyzed how the urinary 8-OH-dG and m^7Gua levels are related to various lifestyle factors. In the univariate analysis of the urinary 8-OH-dG level by the lifestyle and demographic variables, we found a decrease in the urinary 8-OH-dG level with fruit consumption and daily physical activity. Many studies have shown significant relationships between the dietary consumption of fruits and vegetables and the low urinary excretion of 8-OH-dG,[7,32,33] although other studies found no associations between fruits and vegetables and 8-OH-dG.[34,35]

According to Kendall's rank correlation coefficients (Table 2), the urinary 8-OH-dG level was inversely correlated with the total energy consumed. On the other hand, factors positively related to the urinary 8-OH-dG level were cigarettes smoked per day and Brinkman index. Significant relationships between the urinary 8-OH-dG level and cigarette smoking have been observed not only in urine,[17] but also in leukocytes,[36] and lung tissue,[13] However, alcohol consumption was not significantly correlated to urinary 8-OH-dG excretion. Similarly, we did not obtain a significant association between the urinary 8-OH-dG level and the rest index, while good correlations were reported between the urinary 8-OH-dG level and the average number of working hours per day,[37] and the working conditions.[17]

In our previous work,[17,37] and the report by Loft *et al.*[7] there were significant negative correlations between 8-OH-dG and

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Table 3. Multiple regression analysis of log (8-OH-dG) against related factors in 361 male subjects

Independent variables Male subjects ($n = 361$, $R^2 = 0.056$)	Partial r	SE	Beta	P
Physical activity-1	−0.065	0.020	−0.169	*0.001*
Alcohol drinking	0.060	0.020	0.156	*0.003*
Healthy meal combination	−0.065	0.030	−0.117	*0.034*
Intakes of meat, fish, egg, soybean, etc.	−0.066	0.035	−0.100	0.064
Green- and yellow-colored vegetable consumption	0.680	0.038	0.101	0.076

8-OH-dG, 8-hydroxydeoxyguanosine. Note: Statistical analysis was conducted by a stepwise multiple regression analysis. Partial r indicates partial regression coefficient. Beta indicates standardized partial regression coefficient.

Table 4. Multiple regression analysis of log (m^7Gua) against related factors in 361 male subjects

Independent variables Male subjects ($n = 361$, $R^2 = 0.196$)	Partial r	SE	Beta	P
Smoking	0.070	0.001	0.281	*<0.001*
Age	0.080	0.001	0.281	*<0.001*
BMI	−0.012	0.005	−0.125	*0.010*
Dietary index score	−0.010	0.005	−0.113	*0.026*
Frequency of holiday	−0.069	0.036	−0.092	0.058

m^7Gua, 7-methylguanine. Note: Statistical analysis was conducted by a stepwise multiple regression analysis. Partial r indicates partial regression coefficient. Beta indicates standardized partial regression coefficient.

BMI. In the present study, the same tendency was observed in the univariate analysis ($r = -0.065$, $P = 0.066$) (Table 2), but a significant association was not observed in the multiple regression analysis. This discrepancy may be due to differences in statistical calculation methods and in other lifestyle and demographic factors between the present and previous studies. Our present results are consistent with those reported by Pilger *et al.*[38]

In the multiple regression analysis (Table 3), physical activity-1, which includes physical activity by working, showed a strong negative correlation to the urinary 8-OH-dG. This is in good agreement with our previous findings that physical exercise reduced the 8-OH-dG levels in rat organs (liver, lung and heart),[39] human urine,[17] and human leukocyte,[40] although high-intensity exercise has been shown to increase 8-OH-dG excretion.[41–43] Alcohol drinking also correlated with the 8-OH-dG level in the multiple regression analysis. Many studies have shown a significant relationship between alcohol consumption and 8-OH-dG generation in peripheral leukocytes,[44,45] esophageal tissues,[46] liver,[47] and urine.[48,49] Cigarette smoking was not related to the urinary 8-OH-dG level, whereas it was correlated with the urinary 8-OH-dG level, according to the calculation with continuous variables (Kendall's correlation coefficients, Table 2). The discrepancy between the current results and those from other investigations can be explained by variations in sample size, sample composition, methods of urinary 8-OH-dG measurement and statistical methods.

In terms of urinary m^7Gua measurement results, the categorical lifestyle item related to the elevation of urinary m^7Gua excretion was the intake of meat, fish and other protein-rich foods. It is possible that N-nitroso compounds that methylate DNA are produced by the consumption of these foods.[50,51]

In the analysis of continuous variables, age, cigarette smoking and Brinkman index were positively correlated with the urinary m^7Gua level, whereas weight, BMI, total energy consumed, and total meal index score were negatively correlated to the amount of m^7Gua. Particularly, the multiple regression analysis showed that cigarette smoking, age, BMI and meal index score were related to the urinary amount of m^7Gua. These factors explain 19.6% of the entire variation. The inverse correlation between m^7Gua and BMI can be explained by the fact that m^7Gua is a marker of the metabolic rate, and it is lower in people with a high BMI, mainly due to the lower physiological production of heat to maintain body temperature.[52]

Previous studies have shown the strong link between cigarette smoking and the urinary m^7Gua level. For instance, methylated DNA adducts were detected in animal and human tissues, as a result of exposure to tobacco smoke.[19,53] In other studies, the urinary excretion of m^7Gua was shown to be higher in smokers than in non-smokers.[29] Furthermore, the m^7Gua level in human urine decreased after smoking cessation.[54] Therefore, our results confirmed those of previous studies. Moreover, considering that tobacco-specific nitrosamines are a group of carcinogens present in tobacco smoke,[55] the urinary m^7Gua level can be analyzed to monitor DNA methylation and to assess the risk of lung cancers. The measurment of urinary m^7Gua levels would be useful not only to assess the harmful effects of smoking, but also the effects of environmental tobacco smoke.

The present analyses revealed that the urinary m^7Gua level was linked to age, food-related items, such as meat intake, weight, BMI, total energy consumed, and the meal index score. With respect to the effect of age, m^7Gua may be increased due to lower glutathione (GSH) concentration in aged people,[56] because GSH may be involved in scavenging alkylating agents. Ames and collaborators[57] reported that the m^7Gua levels in rat liver DNA were increased 2.5-fold in old rats (24 months old) as compared to the levels in young rats (6 months old). Our results are compatible with their data. It has been argued that age can affect the overall DNA repair capacity. Thus, the amount of m^7Gua in DNA could reflect a balance between methylating stress and DNA repair activity. However, urinary m^7Gua may be related to the total amount of m^7Gua released from DNA, by repair and by spontaneous depurination due to the labile glycosylic bond.

In our study, the creatinine value was used to normalize the urinary m^7Gua level. Urinary creatinine excretion is influenced by muscle mass. This may be a possible explanation for the higher levels of m^7Gua normalized to creatinine with increasing age. To clarify this point, we conducted a correlation analysis between creatinine and age. Although significant associations were obtained not only between age and m^7Gua, but also between age and creatinine, the association between age and m^7Gua ($r^2 = 0.08$) was stronger than the association between age and creatinine ($r^2 = 0.01$). Considering statistical values (r^2), we decided that the present results are not remarkably influenced by the association between age and creatinine.

We also found a significant correlation between the 8-OH-dG and m^7Gua concentrations when the Pearson's correlation coefficient was calculated ($r = 0.122$, $r^2 = 0.015$, $P = 0.02$). This may be explained by the fact that some factors, such as energy consumed, physical activity and smoking, have similar effects on the 8-OH-dG and m^7Gua levels (Table 2). However, the

coefficient of determination (r^2) explains only 1.5% of the entire variation. Therefore, 8-OH-dG and m^7Gua can be considered as independent markers affected by many factors.

The present study suggested that the amount of m^7Gua excreted in the urine is a very sensitive marker in response to aging and lifestyle, such as smoking or dietary habits. Lifestyle has a more significant effect on urinary m^7Gua than 8-OH-dG, based on all statistical analyses. The urinary excretion of m^7Gua has not been extensively investigated as a biomarker,[58] except for the influences of smoking.[29,54] In this study, urine samples from male working subjects at a specific company were analyzed. In the future, in order to prove the usefulness of urinary m^7Gua as a biomarker, we should confirm the reliability and validity of the present findings, according to appropriately designed large-scale studies.

We demonstrated that urinary m^7Gua is a useful biomarker for DNA methylation in humans, in addition to 8-OH-dG, a form of oxidative DNA damage. The urinary m^7Gua excretion value can be a useful marker not only for the assessment of lung cancer risk, but also for evaluating the aging process and various lifestyles.

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Original Contribution

Urea, the most abundant component in urine, cross-reacts with a commercial 8-OH-dG ELISA kit and contributes to overestimation of urinary 8-OH-dG

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ABSTRACT

Urinary 8-OH-dG is commonly analyzed as a marker of oxidative stress. For its analysis, ELISA and HPLC methods are generally used, although discrepancies in the data obtained by these methods have often been discussed. To clarify this problem, we fractionated human urine by reverse-phase HPLC and assayed each fraction by the ELISA method. In addition to the 8-OH-dG fraction, a positive reaction was observed in the first eluted fraction. The components in this fraction were examined by the ELISA. Urea was found to be the responsible component in this fraction. Urea is present in high concentrations in the urine of mice, rats, and humans, and its level is influenced by many factors. Therefore, certain improvements, such as a correction based on urea content or urease treatment, are required for the accurate analysis of urinary 8-OH-dG by the ELISA method. In addition, performance of the ELISA at 4°C reduced the recognition of urea considerably and improved the 8-OH-dG analysis.

8-Hydroxy-2′-deoxyguanosine (8-OH-dG; also called 7,8-dihydro-8-oxo-dG) is one of the major types of oxidatively damaged DNA and is often analyzed as a marker of physiological and nonphysiological, i.e., pathological, oxidative stress [1,2]. It was discovered in 1983 during a study of DNA modifications in vitro caused by mutagens produced by heating carbohydrates [3]. In 1986, Floyd et al. developed a sensitive method to analyze 8-OH-dG, using an electrochemical detector with high-performance liquid chromatography (HPLC-ECD) [4]. Many oxygen radical-forming carcinogens were found to increase 8-OH-dG in cellular DNA by this method [5]. Ames and his collaborators discovered 8-OH-dG excretion into animal and human urine by an HPLC-ECD method [6]. 8-OH-dG may be a good marker for monitoring cellular oxidative stress, which is involved in the induction of cancer and lifestyle-related diseases, and its prevention by antioxidants. 8-OH-dG has been analyzed by ELISA (enzyme-linked immunosorbent assay) and LC-MS-MS (liquid chromatography with electrospray tandem mass spectrometry) methods, in addition to the HPLC-ECD method [7–9]. Among them, the ELISA method is most often used for urinary 8-OH-dG analysis, because an analysis kit is commercially available. However, discrepancies have been observed between the data obtained by the ELISA and HPLC methods [8,10]. Generally the level of disagreement has been more than twofold but, of late, levels have come closer, perhaps owing to refinements in the kit and advice from previous papers on strict temperature control. A good correlation was obtained between the two methods when an HPLC-purified 8-OH-dG fraction was analyzed by the ELISA method [10]. These results suggest that the monoclonal antibody for 8-OH-dG (N45.1) is not sufficiently specific for urinary 8-OH-dG detection and may cross-react with other urinary components. Cooke et al. recently reported that the data of urinary or salivary 8-OH-dG analyzed by ELISA did not correlate with the 8-OH-dG data exactly analyzed by an isotope-dilution LC-MS-MS method [11]. They raised the question about the ability of ELISA approaches to specifically determine the absolute levels of 8-OH-dG in urine and saliva.

In contrast, Toyokuni et al. pointed out that the antibody (N45.1) is highly specific to 8-OH-dG [7]. They confirmed that it recognizes both the 8-hydroxyguanine and the 2′-deoxyribose moieties of the 8-OH-dG molecule. Namely, the ribonucleosides, 8-hydroxyguanosine and 8-mercaptoguanosine, weakly cross-react with N45.1. None of the normal four deoxynucleosides, as well as deoxyuridine, deoxyinosine, O-6-methyldeoxyguanosine, and 8-hydroxydeoxyadenosine; the ribonucleosides of guanosine, 6-mercaptoguanosine, 8-bromoguanosine, and 7-methylguanosine; and the free bases of guanine, 8-hydroxyguanine and O-6-methylguanine; cross-reacted with N45.1. They also reported that urine components such as uric acid, urea, creatine, and creatinine, which are present in urine at rather high concentrations, showed no cross-reactivity with N45.1.

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Despite the long discussion about the 8-OH-dG data discrepancy between the ELISA and the HPLC methods and the overestimation of 8-OH-dG by the ELISA method, the causes of these problems have not been clarified yet. More than 500 papers have been published on urinary 8-OH-dG as a marker of oxidative stress. In many of those studies, the ELISA method was used. If the ELISA kit recognizes urine components other than 8-OH-dG, then it may yield misleading results and interrupt scientific progress in the field of free radical biology. As one approach to clarifying these problems, we fractionated a human urine sample by HPLC and examined the urine components that cause competitive inhibition in the ELISA.

Materials and methods

Materials

Urea and uric acid, and allantoin, were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and Sigma Chemical Co. (St. Louis, MO, USA), respectively. 8-Hydroxyguanine was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). 8-OH-dGMP was prepared as described previously [12]. The 8-OH-G ribonucleoside used for competitive inhibition in the ELISA at 4°C was highly purified by preparative HPLC, as described previously [13], and was crystallized from a 20% aqueous ethanol solution.

Analysis of 8-OH-dG by the ELISA method

Urine samples were collected from 10 men and 13 women (ages 25–61). Urine samples were centrifuged at 2000 *g* for 15 min, and a 50 μl aliquot of the supernatant was used for the 8-OH-dG analysis with a commercial ELISA kit (8-OHdG Check; Japan Institute for the Control of Aging (JICA), Fukuroi, Japan). Although there are other kits available on the market, we have tested the kits from only this company. The incubation with the primary antibody (N45.1) was performed at 37°C, according to the manufacturer's instructions. The 8-OH-dG values from the samples were calculated based on calibration sigmoid plots of the absorbance (450 nm) of an 8-OH-dG standard at various concentrations.

To examine the ELISA method at 4°C, urine samples were collected from 54 men (ages 27–63). The Highly Sensitive 8-OHdG Check ELISA Kit (JICA), which was commercially available from the same company for the assay at 4°C, was used for these analyses. The urine samples were diluted threefold, as recommended by JICA, because without dilution, many urine samples showed irreproducible values near the saturated region in the sigmoid calibration curve.

Analysis of 8-OH-dG by the HPLC method

Urinary 8-OH-dG was determined by the method previously described [13]. Namely, a human urine sample was mixed with the same volume of a dilution solution containing the ribonucleoside marker, 8-hydroxyguanosine (8-OH-G). A 20 μl aliquot of the diluted urine sample was injected into HPLC-1 (MCI GEL CA08F, 7 μm, 1.5×120 mm; elution, 2% acetonitrile in 0.3 mM sulfuric acid, 50 μl/min, 65°C), via the guard column (1.5×40 mm), and the chromatograms were recorded by a Gilson UV detector (UV/Vis-155 with a 0.2-mm light-path cell). The 8-OH-dG fraction was collected, depending on the relative elution position from the peak of the added marker, 8-OH-G, and was automatically injected into the HPLC-2 column. The 8-OH-dG fraction was fractionated by the HPLC-2 column (Shiseido, Capcell Pak C18, 5 μm, 4.6×250 mm; elution, 10 mM sodium phosphate buffer (pH 6.7) containing 5% methanol and an antiseptic reagent, MB (100 μl/L), 1 ml/min, 40°C). The 8-OH-dG was detected by a Coulochem II EC detector (ESA, USA) with a guard cell (5020) and an analytical cell (5011) (applied voltage: guard cell, 350 mV; E1, 170 mV; E2, 300 mV).

Determination of urinary urea

For quantitative colorimetric urea determination, the Quanti-Chrom Urea Assay Kit (DIUR-500; BioAssay Systems, Hayward, CA, USA) was used.

Fractionation of human urine by HPLC

A 3 ml portion of human urine (male, age 61) was concentrated to dryness by a centrifugal concentrator apparatus, CC-101 (Tomy Seiko Co. Ltd., Tokyo, Japan). The residue was dissolved in 1 ml of 3% aqueous methanol. After centrifugation, the supernatant was injected into a reverse-phase column (Capcell Pak C-18 MG; particle size, 5 μm; column size, 10×250 mm; Shiseido Fine Chemicals, Tokyo, Japan; elution, 0–40 min, linear gradient of methanol (3–23%); 40–55 min (100% methanol); elution speed, 3 ml/min) connected to a photo-diode array detector (Hewlett–Packard 1100 HPLC detection system). UV profiles were recorded by monitoring the UV absorbance at 280 nm. The same conditions were used to determine the elution positions of 8-OH-Gua, 8-OH-dGMP, and 8-OH-dG. To determine the elution positions of urea and allantoin, the UV absorbance at 215 nm was monitored. For the ELISA, the eluent was fractionated into 27 fractions (Fr. 1–26, each 4.5 ml, and methanol Fr., 36 ml), which were concentrated to dryness. Each fraction was reconstituted with a much reduced volume of 3% aqueous methanol (0.3 ml) compared to the original urine volume (3 ml) to detect minor cross-reacting compounds by ELISA, even after separated into many fractions.

Urease treatment of urine

Urine samples (0.2 ml) from four men and five women (ages 29–58) were digested with 3.25 units of urease (urease from Jack bean; Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 2 h. Those urine samples were assayed by ELISA after neutralization.

Statistical analysis

The data correlation was tested by a simple linear regression analysis.

Results

Fractionation of human urine and reactivity with N45.1

One of the urine samples was fractionated into 27 fractions by HPLC with a linear gradient of methanol, and each fraction was assayed with the 8-OH-dG ELISA kit (Fig. 1). In addition to the 8-OH-dG fraction (Fr. 16), Fr. 1 (front fraction) and Fr. 27 (methanol fraction) showed positive reactions in the ELISA, as shown in Fig. 1b. We attempted to identify the components in Fr. 1 that react with the ELISA kit. The known urinary components, urea, allantoin, and uric acid, and the 8-OH-dG-related compounds, 8-OH-dGMP and 8-OH-Gua, which were expected to be eluted in the early fractions from the reverse-phase column, were injected into the HPLC system to determine their elution positions. We found that urea and allantoin eluted in Fr. 1 (Fig. 1).

Urea generates a positive reaction with the ELISA method

Urea and allantoin were assayed by ELISA to test whether they competitively inhibit 8-OH-dG recognition by N45.1. As shown in Fig. 2, urea generates a positive reaction in the 8-OH-dG ELISA kit in the range of 10–80 mg/ml. This range is similar to the urea concentration in human urine (15–30 mg/ml) [13]. The correlation between the amounts of 8-OH-dG and urea detected by the ELISA (N45.1) was obtained as shown in Fig. 3, line a. A urea concentration

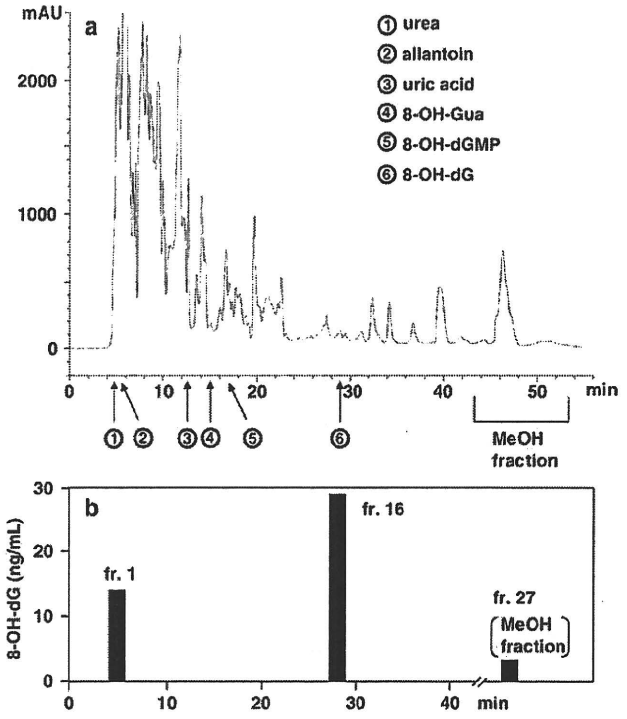

Fig. 1. (a) Fractionation of human urine by reverse-phase HPLC and (b) reactivity to the 8-OH-dG ELISA kit. The elution positions of standard compounds are shown by arrows.

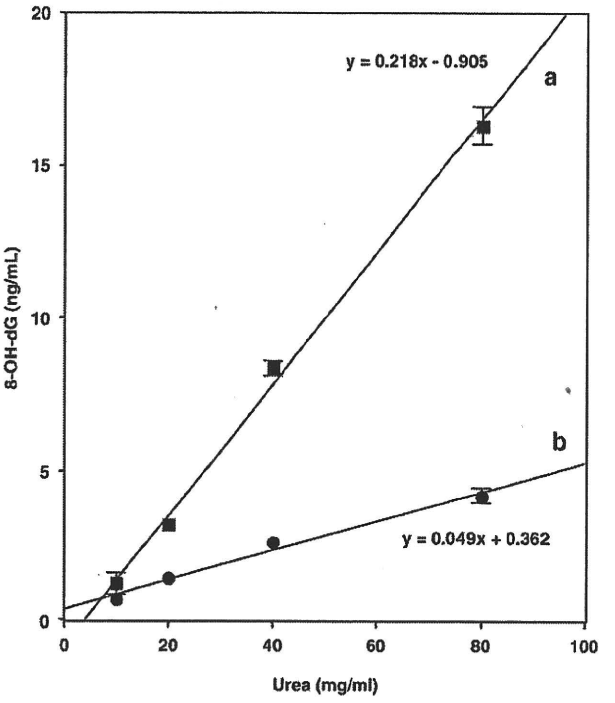

Fig. 3. Comparison of 8-OH-dG and urea amounts detected by the ELISA ($n=3$–6). Mean values ± SD are plotted. Line a, ELISA at 37 °C. Line b, ELISA at 4°C. Absence of SD bars means that the SD values were very low.

of 40 mg urea/ml showed the same competitive inhibition as about 8 ng 8-OH-dG/ml in the ELISA. Therefore, the recognition of urea by N45.1 is about 5,000,000 times lower than that of 8-OH-dG, by a weight-based comparison (24,000,000 times by molar ratio). Allantoin showed no reactivity in the ELISA (data not shown).

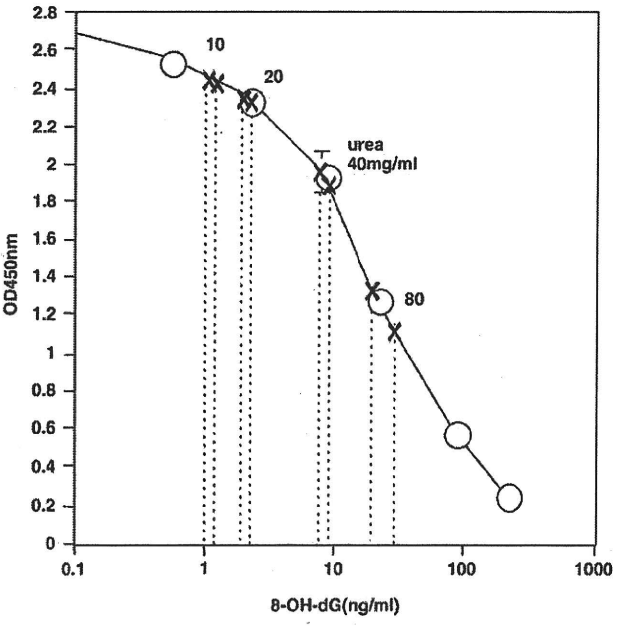

Fig. 2. Competitive inhibition by 8-OH-dG and urea with the 8-OH-dG ELISA kit. (○) 8-OH-dG data (0.5–200 ng/ml); the mean values ± SD are plotted. Absence of SD bars means that the SD values were very low. (×) Urea data (10–80 mg/ml); the mean values are plotted.

Comparison of 8-OH-dG data obtained by the ELISA and HPLC methods before and after urea correction

The 8-OH-dG levels in human urine samples from 23 individuals were analyzed by the ELISA and HPLC methods. Each sample showed a large difference in the 8-OH-dG values obtained by the two methods (Table 1). Higher levels of 8-OH-dG were obtained by the ELISA method in all of the urine samples, compared with those determined by the HPLC method (Fig. 4, closed circles). To calculate how much of the overestimation can be explained by the presence of urea, we determined the concentration of urea in each urine sample (Table 1). According to the correlation equation between urea and 8-OH-dG in the ELISA (Fig. 3, line a), we calculated the equivalent 8-OH-dG concentration due to urea (Table 1). When the HPLC data and the modified ELISA data (ELISA 8-OH-dG data minus the equivalent 8-OH-dG value due to urea) were plotted, all the ELISA data were shifted toward the HPLC values (Fig. 4, open circles). However, with many samples, urea can only partly explain the discrepancies in the data obtained by the two methods. These data suggest that cross-reacting substances other than urea may exist in urine.

To test whether urease treatment can alleviate the overestimation by the ELISA, we treated nine urine samples with urease. We found that most of the samples showed a significant decrease in the 8-OH-dG levels by ELISA after the urease treatment, as shown in Fig. 5.

ELISA at lower temperature

Evans et al. recently reported the utility of an overnight incubation of the primary antibody at 4°C, for lowering the mean urinary 8-OH-dG level [14]. It is reasonable to speculate that the specificities of N45.1 to recognize 8-OH-dG and structurally related compounds may differ, depending upon the assay temperature. Thus, we performed the incubation with the primary antibody (N45.1) at 4°C, overnight. We found that the recognition of urea by the ELISA is reduced at 4°C compared to that at 37°C. A correlation between the amounts of 8-OH-

Table 1
Comparison of 8-OH-dG levels analyzed by ELISA and HPLC methods before and after urea correction.

Sample	Concentration of 8-OH-dG (ng/ml)		Urea (mg/ml)	Equiv. 8-OH-dG conc. due to urea (ng/ml)	Apparent 8-OH-dG conc. (HPLC + urea correction) (ng/ml)
	ELISA	HPLC			
A	2.3	1.1	8.9	1.0	2.1
B	3.1	1.9	11.4	1.6	3.5
C	3.3	1.2	10.3	1.3	2.5
D	4.5	1.7	13.5	2.0	3.7
E	5.8	3.1	12.6	1.8	4.9
F	6.0	4.5	13.4	2.0	6.5
G	7.3	2.2	11.5	1.6	3.8
H	9.8	3.6	14.1	2.2	5.8
I	10.0	3.3	29.7	5.6	8.9
J	12.0	4.5	25.3	4.6	9.1
K	13.2	4.7	19.3	3.3	8.0
L	13.8	5.8	22.7	4.0	9.8
M	16.2	8.3	29.3	5.5	13.7
N	16.5	5.3	27.4	5.1	10.3
O	16.5	5.0	21.4	3.8	8.7
P	20.0	7.9	15.1	2.4	10.2
Q	20.0	8.0	19.0	3.2	11.2
R	23.2	8.2	39.6	7.7	15.9
S	23.3	5.5	18.8	3.2	8.7
T	23.3	6.9	23.6	4.2	11.1
U	26.5	6.9	21.5	3.8	10.6
V	26.5	7.2	25.4	4.6	11.8
W	27.5	7.6	23.1	4.1	11.8

dG and urea detected by the ELISA (N45.1) at 4°C was obtained, as shown in Fig. 3, line b. We then analyzed 54 human urine samples by ELISA at 4°C and HPLC-ECD. Even at the lower temperature, the urinary 8-OH-dG levels measured by ELISA were 1.5-fold higher than those measured by HPLC-ECD (Fig. 6). We suspected that other cross-reacting substances may exist in the urine and found that the ribonucleoside 8-OH-G considerably cross-reacts with N45.1 at 4°C, in addition to urea (Fig. 7). An only 7-fold higher concentration of 8-OH-G over 8-OH-dG was required for the same competitive inhibition in the ELISA.

Discussion

Our data show that one of the major cause of the overestimation of urinary 8-OH-dG by ELISA is the presence of urea in urine. Urea is the most abundant component in urine, and 15–30 g of urea is excreted into human urine per day [15]. 8-OH-dG (8-oxo form) and urea share a common –NH–CO–N– structure, as shown in Fig. 8. This may be the reason urea is weakly recognized by N45.1. There was a 5,000,000-fold difference based on weight (24,000,000 times based on molar ratio) in the affinity of N45.1 between urea and 8-OH-dG. Toyokuni et al. reported that urea showed no cross-reactivity with N45.1 [7]. However, they tested only 10^{-3}–10^{2} μM concentrations of urea, which are 4 orders of magnitude lower than those in human urine. Urea is excreted into urine by the decomposition of protein [16] and is not directly related to oxidative stress. For example, in many epidemiological studies, diet influenced 8-OH-dG levels, suggesting that the food components or antioxidants induce or reduce oxidative stress [17]. However, a protein-rich diet may cause an increase in the urinary excretion of urea, and thus the 8-OH-dG levels will be overestimated

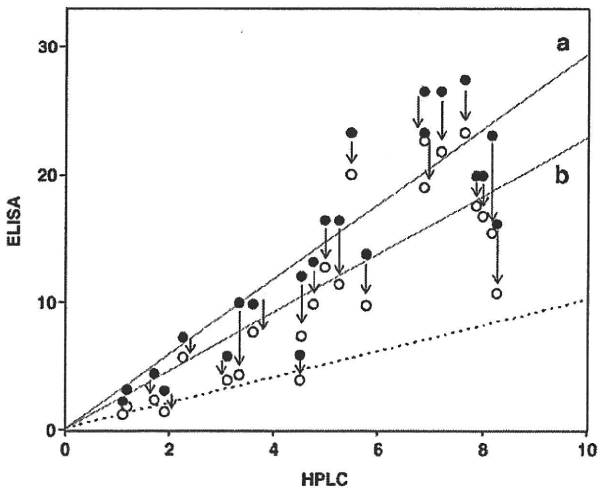

Fig. 4. Comparison of 8-OH-dG levels (ng/ml) determined by ELISA (37°C) and HPLC methods before and after urea correction. (●) Plot of raw ELISA vs HPLC data. Line a, $y = 2.93x$, $r = 0.973$, $p < 0.0001$. (○) Plot of ELISA data minus equivalent 8-OH-dG value due to urea vs HPLC data. Line b, $y = 2.28x$, $r = 0.953$, $p < 0.0001$. Arrows indicate shift after urea correction. The dotted line corresponds to full concordance between the two methods.

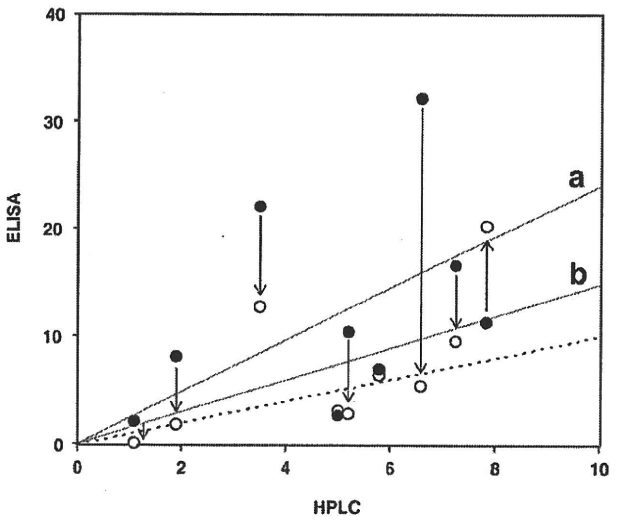

Fig. 5. Comparison of 8-OH-dG levels (ng/ml) determined by ELISA (37 °C) and HPLC methods before and after urease treatment. (●) Before urease treatment. Line a, $y = 2.41x$, $r = 0.832$, $p = 0.0028$. (○) After urease treatment. Line b, $y = 1.48x$, $r = 0.859$, $p = 0.0015$. Arrows indicate shift after urease treatment.

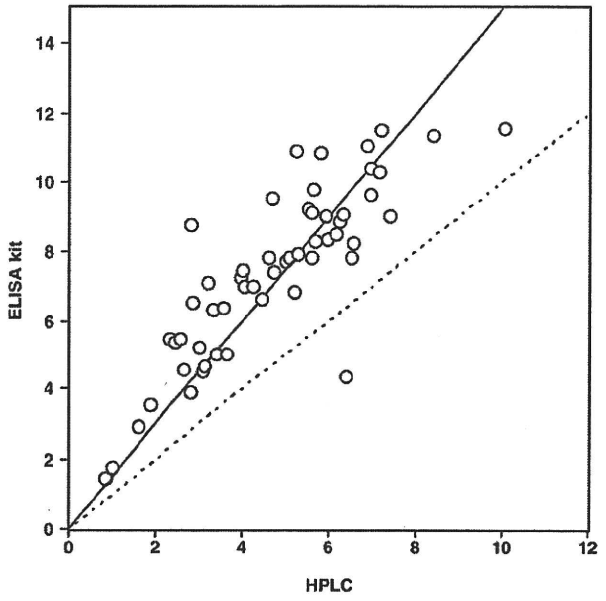

Fig. 6. Comparison of urinary 8-OH-dG levels (ng/ml) determined by ELISA (4°C) and HPLC methods. $y = 1.50x$, $r = 0.980$, $p < 0.0001$.

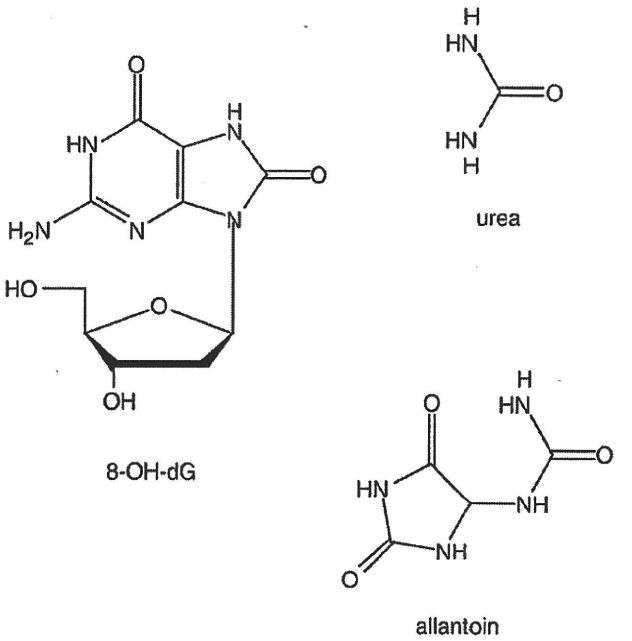

Fig. 8. Structures of 8-OH-dG (8-oxo form), urea, and allantoin.

when the ELISA method is used for 8-OH-dG determination. All of those studies should be reevaluated by accurate measurements using HPLC, or at least the urea concentrations in these samples should be examined for their final conclusions. Hu et al. reported the analysis of 8-OH-dG in 140 urine samples collected from workers in a coke oven plant, by both LC-MS-MS and ELISA methods [18]. Only the LC-MS-MS measurements of 8-OH-dG showed a significant difference between the exposed and the control subjects. When we analyzed 120 urine samples, 10% of the samples showed an ELISA:HPLC ratio higher than 4 [10]. Those subjects might have a high urea concentration. Therefore, the ELISA method may generate a misleading conclusion and, at present, cannot be used for clinical analyses. This is in agreement with the current recommendations of the European Standards Committee on Urinary Lesion Analysis (ESCULA) [19].

In this study, when we measured the urinary urea concentration with a commercial kit, the urea accounted for only 34% of the overestimation by the ELISA (Fig. 4). However, based on the experiments with the urease treatment, 66% of the overestimation could be explained by urea (Fig. 5). One possible explanation for this discrepancy may be as follows. The measurement of urinary urea is underestimated, because a portion of the urea is weakly bound to other urinary components and is not detected by the analysis kit, whereas the urease treatment revealed the total amount of urea, including the bound form. In this hypothesis, the bound form of urea must be recognized in the ELISA.

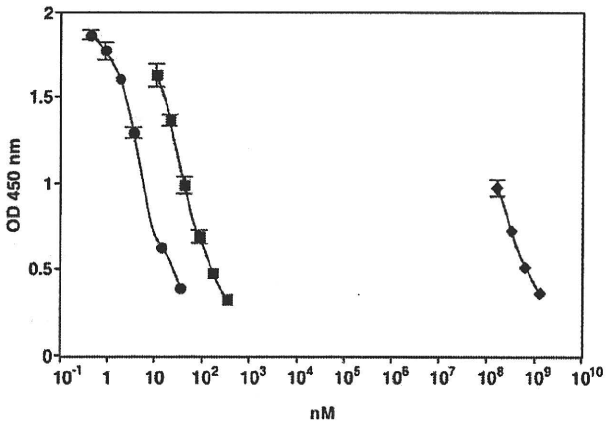

Fig. 7. Competitive inhibition of ELISA at 4 °C by 8-OH-dG (●), 8-OH-G (■), and urea (♦). Mean values ± SD are plotted ($n = 3$–6). Absence of SD bars means that the SD values were very low.

It will be suspected that other urea derivatives, such as citrulline and biuret, cross-react with N45.1, in addition to urea. However, they may not contribute much to the overestimation of urinary 8-OH-dG, because their urinary contents may be very low. For example, the urinary citrulline level is on the order of micrograms per milliliter [20]. The methanol fraction (Fr. 27) in the HPLC analysis also contained a small amount of components that showed a positive reaction in the ELISA system (Fig. 1). This fraction may contain short DNA fragments containing 8-OH-dG, as suggested by Lunec and his collaborators [21].

It is possible that the concentrations of urea used in the competitive ELISA are denaturing the N45.1, or interfering with N45.1 binding, rather than acting as a competitive inhibitor. However, protein denaturation usually occurs with a high concentration of urea, such as 5–8 M, whereas the urinary urea concentration is below 1 M. Therefore, the denaturation of an antibody by urinary urea is unlikely. In addition, many compounds in urine are analyzed by ELISA without such problems. Therefore, we prefer the idea that urea is weakly recognized by N45.1 owing to structural similarity, which induces competitive inhibition. In either case, the recognition of urea by N45.1 and the denaturation of N45.1 would both have similar consequences when reading the ELISA plate, but perhaps this is outside the scope of this article.

We found that performing the ELISA at 4°C reduced the recognition of urea and improved the 8-OH-dG analysis by ELISA. However, at 4°C, the ribonucleoside 8-OH-G, which has a source different from that of 8-OH-dG, cross-reacted with the ELISA in addition to urea. Toyokuni et al. [7] reported that 8-OH-G needs to be present in a concentration 2 orders of magnitude higher than that of 8-OH-dG to compete to the same extent in the ELISA at 37°C. Although Evans et al. [14] reported that 8-OH-G needs to be present at 25 times the concentration of 8-OH-dG to exert equal competition at 4°C, in our experiments, an 8-OH-G concentration only 7-fold higher than that of 8-OH-dG showed the same competition. Considering that Weimann et al. reported that the 8-OH-G levels in human urine are about 2 times higher than the 8-

OH-dG levels [22], 8-OH-G may also contribute considerably to the overestimation of 8-OH-dG by the ELISA at 4°C.

In conclusion, urea, the most abundant component in urine, was found to cross-react with a commercial 8-OH-dG ELISA kit and to cause the overestimation of 8-OH-dG. Urea is present in high concentrations in the urine of humans and experimental animals, and its level is influenced by many factors other than oxidative stress. Therefore, improvements such as urea removal by pretreatment or its enzymatic decomposition by urease are required for the measurement of urinary 8-OH-dG by the ELISA at 37°C. Performing the ELISA at 4°C reduced the recognition of urea and improved the 8-OH-dG analysis. It is more desirable that a monoclonal antibody that specifically recognizes 8-OH-dG is developed in the future.

Acknowledgments

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性腺・その他

長期経過中に多彩な合併症を呈した McCune-Albright syndrome の1例

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はじめに

McCune-Albright Syndrome（以下 MAS と略）は，線維性骨異形成症（fibrous dysplasia；以下 FD と略），皮膚の色素沈着（カフェオレ斑），内分泌異常（思春期早発症，甲状腺機能亢進症，先端巨大症やクッシング症候群など）を3主徴とする症候群である．本症では発生過程において，体細胞レベルで促進性 G 蛋白 α subunit（以下 Gs α と略）遺伝子の点突然変異が起き，細胞内の cAMP 濃度が上昇して内分泌機能亢進や細胞増殖を引き起こす．

今回我々は20年前に3主徴より MAS と診断され，経過中に膵嚢胞性病変，多発性大腸ポリープ，大腸腺腫内癌，筋萎縮性側索硬化症を合併した症例を経験したので報告する．

症　例

症　例　56歳，男性．

主　訴　嚥下障害と筋力低下．

既往歴　特記事項なし．

家族歴　母に糖尿病．

現病歴　28歳時（1976年）に甲状腺腫に気付いたが放置し，徐々に増大した．36歳時（1984年）に6カ月で7kgの体重減少，胸部不快感，全身倦怠感を自覚したため受診した．血液検査では T_4 17.9 μg/dl（正常 6.0～12.0 μg/dl），T_3 196 ng/dl（正常 90～180 ng/dl），TSH 1.1 μU/ml（正常＜8 μU/ml）と甲状腺機能亢進がみられ（**表1**），TRH 負荷試験で TSH の上昇はみられなかった（**表2**）．甲状腺超音波検査では右葉外側に4 cm 大の嚢胞と，左葉に多数の充実性腫瘤および嚢胞を認めた．甲状腺シンチでは ^{123}I 摂取率は亢進し，左葉上極に hot nodule を認めた（**図1**）．以上より機能性腺腫様甲状腺腫と診断され，thiamazole 投与にて治療された．眉弓部突出，頭蓋変形がみられ，血中 GH 20 ng/ml，IGF-1 3.21 U/ml（正常 0.36～2.00 U/ml）と高値であり，ブドウ糖負荷にて血中 GH は抑制されず，血糖は2時間値 220 mg/dl と糖尿病型であった．GH は GRH にて上昇したが，TRH，LHRH に対する奇異性反応はみられなかった（**表1, 2**）．頭部 CT では下垂体に腺腫はみられなかった．以上より先端巨大症と診断された．頭部は大きくいびつで，頭部 XP では頭蓋骨の不規則な肥厚による変形，骨シンチでは頭蓋骨と左鎖骨に集積を認め（**図2**），頭蓋の骨生検より線維性骨異形成症を認めた．下顎に色素沈着あり，内分泌異常，

表1　初診時検査所見（1984年）

Ulynalysis		Blood chemistry			Endocrine data		
Protein	+	TP	6.0	g/dL	T_3	196 ng/dL	(90～180)
Glicose	2 +	Alb	3.6	g/dL	T_4	17.9 μg/dL	(6.0～12.0)
Ketone	−	BUN	13	mg/dL	TSH	1.1 μU/mL	(＜8)
Occ. bl	−	Cre	0.6	mg/dL	TG Ab	−	
Urobil	±	Na	143	mEq/L	MC Ab	−	
		K	3.8	mEq/L	GH	20 ng/mL	(5以下)
Peripheral Blood		Cl	107	mEq/L	IGF-1	3.21 U/mL	(0.36～2.0)
WBC	4450 /mL	Ca	8.9	mg/dL	PRL	50 ng/mL	(30以下)
RBC	483×10^4 /mL	P	3.8	mg/dL	GHRH	5 pg/mL	(3.9～11.7)
Hb	13.8 g/dL	AST	14	IU/L	LH	10.2 IU/L	
Ht	32.2 %	ALT	22	IU/L	FSH	16.1 IU/L	
PLT	26.9×10^4 /mL	LDH	179	IU/L	ACTH	54 pg/mL	
		ALP	135	IU/L	Cortisol	11.3 μg/dL	
		T-Cho	102	mg/dL	U-Ad	14.8 μg/day	
		TG	48	mg/dL	U-Norad	42.8 μg/day	
		Glu	103	mg/dL	U-Dopa	486.4 μg/day	

表2　内分泌学的負荷テスト（1984年）

75g OGTT					
時間（分）	0	30	60	90	120
BS（mg/dL）	116	153	133	201	220
IRI（μU/mL）	9.6	18.1	25.5	26	29.3
GH（ng/mL）	20	10	13	15	14
GRH負荷					
時間（分）	0	30	60	90	120
GH（pg/mL）	14	29	36	20	19
TRH負荷					
時間（分）	0	30	60	90	120
TSH（μU/mL）	1.1	1.3	1.1	1以下	1以下
GH（pg/mL）	13	11	17	12	12
PRL（ng/mL）	60	46	53	48	51
LHRH負荷					
時間（分）	0	30	60	90	120
LH（mIU/mL）	10.2	175.5	195.2	153.2	127.2
FSH（mIU/mL）	16.1	51.4	64.2	63.2	64.2
GH（pg/mL）	13	14	20	13	12
PRL（ng/mL）	50	49	57	48	55

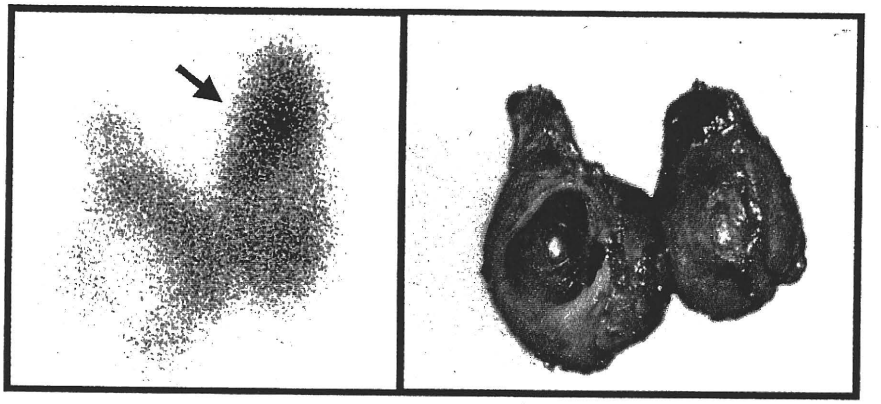

図1　機能性腺腫様甲状腺腫

左：甲状腺シンチグラムでは Thyroidal I^{123} uptake ; 24 hr 58. 3 %と取り込みは亢進し，右葉下極に cold nodule，左葉上極に hot nodule（矢印）がみられた．
右：甲状腺摘出標本．

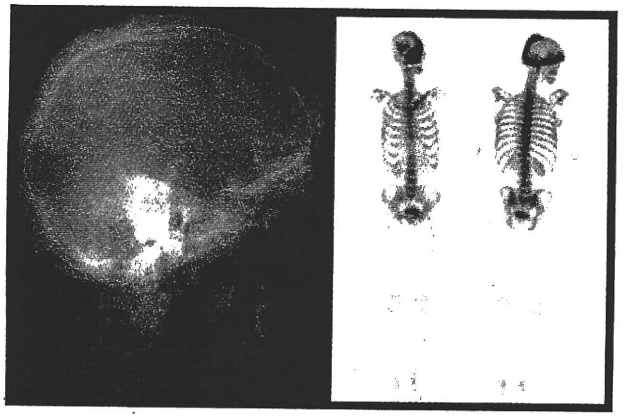

図2　多発性線維性骨異形成症

左：頭部 XP では頭蓋骨の不規則な肥厚による変形を認める．
右：骨シンチ検査では頭蓋骨および左鎖骨に著明な hyperactivity を認める．

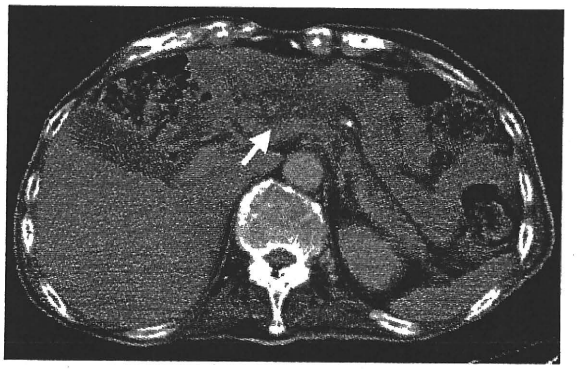

図3　膵嚢胞性病変

腹部 CT では，膵頭部より体部にかけ多発性嚢胞性病変と主膵管の拡張（矢印）がみられた．

線維性骨異形成症の3主徴がみられることよりMcCune-Albright Syndrome と診断された[1]．46歳時に気管圧排のため甲状腺全摘され，摘出標本（図1）の組織診断は腺腫様甲状腺腫であった．甲状腺に Gs α 遺伝子の Arg201（→ His）変異が確認された．また，腹部 CT にて膵頭部より体部にかけ多発性嚢胞性病変と主膵管の拡張を認めたが（図3），消化管ホルモン値は正常範囲であった．ERCP 下で膵液より採取された細胞診で異型は認められなかった．55歳時に多発性大腸ポリープを認め内視鏡的切除術が施行された．採取されたポリープ5個のうち1個に大腸腺腫内癌が認められた（図4）．膵液細胞，大腸ポリープの遺伝子検査では Gs α 遺伝子変異は認めなかった．同時期より嚥下障害，舌萎縮，両手筋萎縮が出現し，嚥下障害進行したため56歳時に胃ろうを造設した．また，誤嚥性肺炎を起こし喀痰排出困難のため気管切開が施行された．徐々に左右非対称

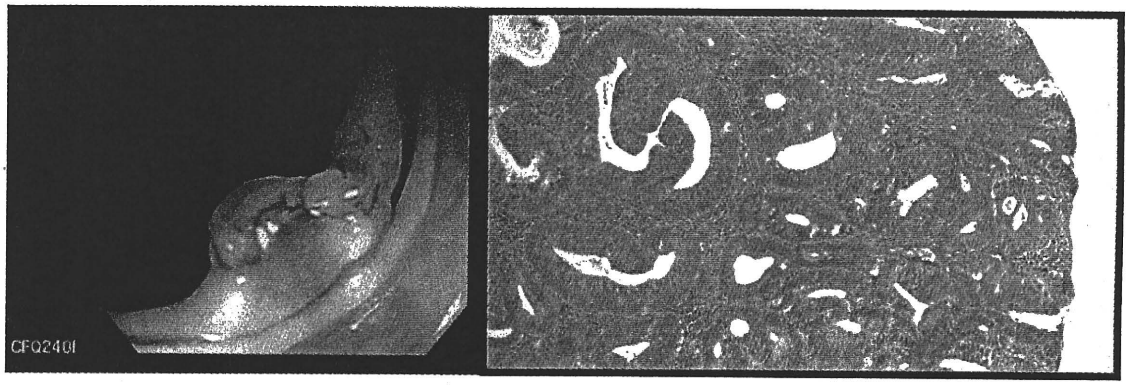

図4　大腸腺腫内癌

左：大腸内視鏡検査ではS状結腸にIp，直径8 mm大のポリープを認めた．
右：生検組織ではwell differentiated adenocarcinomaを認めた．

表3　入院時検査所見

Peripheral Blood		Blood chemistry		Endocrine data	
WBC	5900 /μL	TP	6.9 g/dL	fT_3	3.87 pg/mL
RBC	367 × 10^4 /μL	Alb	2.68 g/dL	fT_4	1.28 ng/dL
Hb	10.5 g/dL	BUN	13.8 mg/dL	TSH	3.87 μIU/mL
Ht	32.2 %	Cr	0.24 mg/dL	GH	7.32 ng/mL
PLT	26.9 × 10^4 /μL	Na	136 mEq/L	IGF-1	190 ng/mL
		K	3.9 mEq/L	ACTH	42.1 pg/mL
Immunological test		Cl	98 mEq/L	Cortisol	16.0 μg/dL
IgG	1422 mg/dl	AST	18 IU/L	Aldosterone	186 pg/mL
IgA	373 mg/dl	ALT	23 IU/L	PRA	8.5 ng/mL/時間
IgM	119 mg/dl	ALP	132 IU/L	Ad	15 pg/mL
RA	陰性	LDH	149 IU/L	Norad	199 pg/mL
LE test	陰性	CPK	48 IU/L	Dopamine	10 pg/mL
補体価	51.8 CH_{50}/ml	T-Cho	127 mg/dL		
		TG	96 mg/dL		
		Glu	127 mg/dL		
		HbA1c	7.4 %		
		CRP	0.45 mg/dL		

の上肢優位の筋力低下が進行し起立困難となったため，2005年4月精査加療目的にて当科へ入院した．

入院時現症　身長172 cm，体重44.3 kg，血圧108/78 mmHg，脈拍88/min，体温36.7℃，頭部は大きくいびつで前頭部・側頭部が突出していた．視野正常で眼底に異常なし．左難聴あり．顎に皮膚の色素沈着が認められた．胸，腹部に異常なし．舌と上肢優位の四肢筋に萎縮あり．深部腱反射は上肢で消失し，下肢で亢進していた．感覚障害なく，膀胱直腸機能や眼球運動に異常なし．

入院時検査所見　血液検査では貧血，低蛋白血症，CRPの軽度上昇を認めた．また，血清GHは軽度上昇，免疫学的検査に異常はみられなかった