Life Style and Urinary 8-Hydroxydeoxyguanosine, a Marker of Oxidative DNA Damage: Effects of Exercise, Working Conditions, Meat Intake, Body Mass Index, and Smoking

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The urinary levels of 8-hydroxydeoxyguanosine (8-OH-dG), a marker of oxidative DNA damage, of 318 healthy men aged 18–58 were measured with high resolution by a newly developed automated high-pressure liquid chromatography (HPLC) system coupled to an electrochemical detector (ECD). The mean 8-OH-dG level (µg/g creatinine) was 4.12±1.73 (SD). An eleven-fold inter-individual variation was observed. The accuracy of the measurement estimated from the recovery of an added 8-OH-dG standard was 90–98%. By univariate analysis, it was found that moderate physical exercise (P=0.0023) and high body mass index (BMI) (P=0.0032) reduced the 8-OH-dG level, while physical labor (P=0.0097), smoking (P=0.032), and low meat intake (less than once/week) (P=0.041) increased its level. Based on a multi-regression analysis of the log-transformed values, moderate physical exercise (P=0.0099), high BMI (P=0.0099), and age (P=0.021) showed significant reducing effects on the 8-OH-dG level, while low meat intake (P=0.010), smoking (P=0.013), and day-night shift work (P=0.044) increased its level. These results suggest that many types of life-style factors that either generate or scavenge oxygen radicals may affect the level of oxidative DNA damage of each individual.

Key words: 8-Hydroxydeoxyguanosine — Exercise — Meat intake — BMI — Smoking

Oxygen radicals are formed in cells by endogenous oxygen metabolism, as well as by various environmental agents, and induce damage to cellular macromolecules such as DNA, RNA, and protein.10 It has been suggested that oxidative DNA damage is involved in animal carcinogenesis, either induced spontaneously25 or by a group of chemicals that generate oxygen radicals.31 Many types of oxidative DNA damage have been reported.4 Among them, 8-hydroxydeoxyguanosine (8-OH-dG) is a major type and is frequently analyzed as a marker of cellular oxidative stress relevant to carcinogenesis,33 because 8-OH-dG in DNA induces mutations,6,7 and it is rather easily analyzed by high-pressure liquid chromatography coupled to an electrochemical detector (HPLC-ECD).8 Many oxygen radical-forming carcinogens induce an increase of 8-OH-dG in the target organ DNA in animal experiments.5 In humans, we have observed higher levels of 8-OH-dG in the lungs of smokers,9 the livers of chronic hepatitis patients,10 and the stomachs of patients infected with Helicobacter pylori.11

Based on the hypothesis that one of the main causes of human cancer is oxidative stress induced by various endogenous and exogenous (environmental) factors, it is of interest to measure the urinary 8-OH-dG levels of individuals to evaluate their cancer risk. Although the mechanisms by which 8-OH-dG is excreted in urine, such as by nucleotide excision repair or hydrolysis of the oxidized nucleotide by the sanitization enzyme hMTH1, have not been clearly elucidated, several groups have analyzed urinary 8-OH-dG by HPLC-ECD,12 liquid chromatography-tandem mass spectrometry (LC-MS-MS),13 gas chromatography-mass spectrometry (GC-MS),14 or enzyme linked immunosorbent assay (ELISA).15 It has 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, and higher in men than in women, and that it is negatively associated with body mass index (BMI).2,12 In this study, the urinary 8-OH-dG levels of 318 healthy men were determined by a newly developed high resolution analytical system using a combination of two different types of HPLC columns and an ECD. It was found that various lifestyle factors influence the urinary 8-OH-dG level in different ways.

MATERIALS AND METHODS

Urine collection After informed consent had been obtained, urine samples were collected in paper cups from 318 healthy men aged 18–58 in a steel-manufacturing
company, in the afternoon (12:45–15:30) during their periodic health examination, and aliquots of each urine sample were transferred to Eppendorf tubes (5×1 ml, for each subject) and were kept frozen (−80°C) until the 8-OH-dG analysis. At the same time, individual information on age, height and weight (for BMI), status of cigarette smoking and alcohol drinking, intake of meat and vegetables intake, level of mental stress, hours of sleep, working conditions (physical labor, office work, day and night shift work), physical activity as sport (walking, jogging, baseball, badminton, etc.), family history of disease (cancer, diabetes, and hypertension) was obtained through a questionnaire.

Analysis of 8-OH-dG The urinary 8-OH-dG level was determined using an apparatus in which 1) an autosampler (JASCO AS-950-10), 2) a first HPLC system, 3) an autosampling injector (Gilson, 231 XL), 4) a second HPLC system, and 5) an ECD (Coulochem II, esa; guard cell, 350 mV; channel 1, 300 mV; channel 2, 150 mV) were sequentially connected. One milliliter of each urine sample was defrosted, diluted with 0.5 ml of water, and acidified with 45 µl of acetic acid. After complete mixing, the urine solution was centrifuged (15 000 rpm, 5 min) to remove suspended solids, and 0.75 ml of the supernatant was injected into the first HPLC column [Shodex Asahipak GS-320HQ 7G (500×7.6 mm); elution, 0.1% acetic acid; speed, 1 ml/min; temperature, 25°C]. An aliquot (100 µl) of the fraction containing 8-OH-dG (50–61 min) was automatically injected into the second HPLC column [YMC-Pack ODS-AM (250×4.6 mm); elution, aqueous methanol (5%) containing 35 mM NaOAc, 12.5 mM citric acid, pH adjusted to 7.5 by adding 1 M NaOH solution; speed, 0.8 ml/min, temperature, 25°C]. After fractionation of each urine sample, the first column was washed with methanol, while the second column was continuously eluted by recycling with the same buffer (3 liters). The urine samples were sequentially analyzed with this system. When an impurity peak interfered with the 8-OH-dG peak in the second HPLC system, which occurred in ca. 10% of the samples, the fraction was re-analyzed under different elution conditions, using either the same eluent as above, except that the pH was adjusted to 8.2 instead of 7.5, or 10 mM NaH₂PO₄ containing 5% MeOH, pH 4.9.

Creatinine values in urine were determined by a commercial laboratory using a colorimetric method (BML Corp., Kitakyushu).

Statistics The normality of 8-OH-dG distribution was rejected by the Shapiro-Wilks test ($P=0.0001$, skewness =0.873, kurtosis=1.014). For the logarithm of 8-OH-dG, the normality of the distribution was accepted by the Shapiro-Wilks test ($P=0.09$, skewness =−0.331, kurtosis =0.058). The logarithm of 8-OH-dG was therefore used in the analysis. The continuous variables, BMI, smoking and age, were categorized as follows: BMI ($\leq 21.8$, 21.9–24.4, 24.5–), age ($\leq 30$, 31–42, 43–), smoking (0, 1–19, 20–).

Univariate analysis: For categorical variables, the ANOVA of log(8-OH-dG) against each life style factor was done, and the $P$ values of the difference were calculated. For continuous variables, the association between the factor variable and 8-OH-dG was assessed by Pearson’s correlation coefficient with log(8-OH-dG), and similar ANOVA of log(8-OH-dG) was done.

Multivariate analysis: Exercise, working conditions, and meat intake did not show a linear trend, so we constructed new variables, exercise (<5 h/week), working conditions...
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In order to evaluate the association between 8-OH-dG and life style factors, a multiple regression analysis of log(8-OH-dG) against life style factors was done, where independent variables were selected using the forward variable selection procedure with a level of entry of 0.13. For the selected categorical or categorized variables, the ANOVA of log(8-OH-dG) against each life style factor was adjusted for the selected variables by the forward selection procedure.

RESULTS

Analysis of Urinary 8-OH-dG  Urinary samples were analyzed by a newly developed automated HPLC system composed of two columns and an ECD. The key features

Table I. Geometric Means of 8-OH-dG by Life Style Factors

| Variable          | N    | Geometric mean of 8-OH-dG[^a^] | CI       | P value of the difference |
|-------------------|------|-------------------------------|----------|--------------------------|
|                   |      |                               |          |                          |
| Exercise          |      |                               |          |                          |
| 1. None           | 143  | 4.03                          | 3.75     | 4.33                     | 0.0023 | 0.7607 |
| 2. ≤5 h/week      | 147  | 3.45                          | 3.22     | 3.70                     | 0.0408 |
| 3. ≥5 h/week      | 28   | 4.14                          | 3.50     | 4.89                     |        |
| Working conditions|      |                               |          |                          |
| 1. Sitting        | 135  | 3.61                          | 3.35     | 3.88                     | 0.6042 | 0.0097 |
| 2. Walking        | 129  | 3.71                          | 3.44     | 4.00                     | 0.0293 |
| 3. Physical labor | 54   | 4.32                          | 3.84     | 4.87                     |        |
| Meat intake       |      |                               |          |                          |
| 1. ≤5/week        | 64   | 3.82                          | 3.42     | 4.25                     | 0.5768 | 0.1162 |
| 2. 2–4 times/week | 235  | 3.69                          | 3.49     | 3.90                     | 0.0406 |
| 3. ≤1/week        | 19   | 4.56                          | 3.70     | 5.63                     |        |
| Shift work        |      |                               |          |                          |
| 1. Daytime        | 149  | 3.58                          | 3.34     | 3.84                     | 0.0639 |
| 2. Day-night shift| 169  | 3.92                          | 3.67     | 4.19                     |        |
| Stress            |      |                               |          |                          |
| 1. None           | 39   | 3.50                          | 3.04     | 4.03                     | 0.3375 | 0.1104 |
| 2. Medium         | 247  | 3.76                          | 3.56     | 3.97                     | 0.2503 |
| 3. Strong         | 32   | 4.13                          | 3.53     | 4.84                     |        |
| Diabetes (family) |      |                               |          |                          |
| 1. Yes            | 288  | 3.79                          | 3.60     | 3.99                     | 0.3393 |
| 2. No             | 30   | 3.50                          | 2.97     | 4.12                     |        |
| Vegetables        |      |                               |          |                          |
| 1. Every meal     | 174  | 3.78                          | 3.55     | 4.04                     | 0.7648 |
| 2. Some meals     | 144  | 3.73                          | 3.47     | 4.01                     |        |

[^a^] 8-OH-dG (µg/g creatinine).
CI: confidence interval.

Table II. Association of log(8-OH-dG) with Continuous Variables

| Variable       | N   | Mean   | SD    | Min | Max   | Correlation coefficient | P       |
|----------------|-----|--------|-------|-----|-------|-------------------------|---------|
| BMI            | 318 | 23.416 | 23.416| 15.7| 37.2  | −0.165                  | 0.0032  |
| Smoking[^a^]   | 318 | 15.402 | 15.402| 0   | 40    | 0.120                   | 0.0323  |
| Age[^b^]       | 318 | 36.059 | 10.900| 18  | 58    | −0.078                  | 0.1646  |
| Drinking[^b^]  | 318 | 5.189  | 5.189 | 0   | 31.5  | −0.017                  | 0.7565  |

[^a^] Number of cigarettes smoked per day.
[^b^] Number of glasses drunk per week (converted to Japanese sake).
of this system are 1) the use of two columns, which makes the 8-OH-dG analysis easy and reproducible, while other groups use three columns, and 2) the use of a multi-function HPLC column, Asahipak GS-320HQ, which has the functions of gel filtration-, reverse phase-, and ion exchange-column fractionations, in the first step. By injecting a urine sample into this column, most of the proteins, salts, and low-molecular-weight impurities can be removed simultaneously from the 8-OH-dG fraction, which allows the detection of 8-OH-dG as a single peak in the second HPLC (Fig. 1c). The 8-OH-dG peak was identified by co-elution with an authentic standard (Fig. 1b). The standard curve for 8-OH-dG was linear over the range of the experiments (Fig. 2). The mean 8-OH-dG level (µg/g creatinine) was 4.12±1.73 (SD). An eleven-fold inter-individual variation was observed (1.01–11.09 µg/g creatinine). The accuracy of the measurement, estimated from the recovery of an added 8-OH-dG standard was 90–98%. When the same urine sample was analyzed three times, the variation of the data was within ±7%. The urinary 8-OH-dG level was unchanged, even when urine samples were kept at room temperature for 24 h. This result is in contrast to the situation in DNA analysis, where the 8-OH-dG level readily increases owing to autooxidation of the dG.

### Univariate analysis
The association of the 8-OH-dG level with categorical variables (Table I) or with continuous variables (Table II) was investigated. Regarding age, BMI and smoking, the mean 8-OH-dG levels for the categories are shown in Table III. In terms of the lifestyle information obtained by the questionnaire, moderate physical activity (sports <5 h/week) (P=0.0023) and obese body mass (BMI) (P=0.0032) significantly reduced the 8-OH-dG level, while physical labor (P=0.0097), smoking (P=0.032), and low meat intake (P=0.041) increased its level (Tables I and II). Mental stress (P=0.11) and day-night shift work (P=0.064) showed some increasing effect, although it was not statistically significant (Table I).

### Multivariate analysis
Nine independent variables were selected using a forward selection procedure with the level of entry determined according to a multiple regression analysis of log(8-OH-dG) against life style factors. The selected nine variables and the cumulative R-squares (parentheses) are exercise (0.033), BMI (0.054), working conditions (0.070), meat intake (0.084), smoking (0.095), age (0.106), day-night shift work (0.115), vegetable intake (0.122) and family history of diabetes (0.129). Based on a multiple regression analysis against nine variables, moderate physical exercise (<5 h/week) (P=0.0039), high BMI (P=0.0099), and age (P=0.021) showed significant reducing effects on the 8-OH-dG level (Table IV). Smoking

### Table III. Effects of Age, BMI and Smoking on 8-OH-dG Levels

| Variable | N  | Geometric mean of 8-OH-dG | CI    | P values of the difference |
|----------|----|-------------------------|-------|---------------------------|
|          |    |                         |       | 2 | 3 | |
| Age      |    |                         |       |   |   | |
| 1. –30   | 111 | 4.04                    | 3.73  | 4.38 | 0.0531 | 0.0785 |
| 2. 31–42 | 105 | 3.60                    | 3.32  | 3.92 | 0.8732 |
|          | 102 | 3.64                    | 3.34  | 3.96 |
| BMI      |    |                         |       |   |   | |
| 1. –21.8 | 107 | 4.35                    | 4.01  | 4.72 | 0.0001 | 0.0009 |
| 2. 21.9–24.4 | 107 | 3.42                    | 3.15  | 3.71 | 0.4388 |
| 3. 24.5– | 104 | 3.58                    | 3.30  | 3.88 |
| Smoking  |    |                         |       |   |   | |
| 1. None  | 85  | 3.45                    | 3.14  | 3.78 | 0.2697 | 0.0205 |
| 2. 1–19  | 60  | 3.74                    | 3.35  | 4.17 | 0.4163 |
| 3. 20–   | 173 | 3.94                    | 3.69  | 4.20 |

a) Number of cigarettes smoked per day.
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(P=0.013) and low meat intake (≤1/week) (P=0.010) increased the 8-OH-dG level. Day-night shift work (P=0.044) and physical labor (P=0.058) also showed increasing effects. However, the nine independent life style factors listed in Table IV explain only 13% of the entire variation. Mean 8-OH-dG levels calculated by adjusting for other variables are shown in Table V. These results suggest that many types of oxygen radical-forming life-style factors, most of which are unknown, may additively or synergistically affect the level of oxidative DNA damage of each individual.

**DISCUSSION**

It has been suggested that the urinary level of 8-OH-dG in various animal species correlates with oxygen consumption and the amount of endogenously formed oxygen radicals.17 Oxidative DNA damage, such as 8-OH-dG, may induce cancer, and reduce maximum life span. It is of interest, therefore, to measure its level in human individuals as a predictor for cancer risk.18 if oxygen radicals are one of the main causes of human cancer. However, only three reports have been published thus far on large-scale (n>300) analyses of human urinary 8-OH-dG.15,16,19 In the present study, we found that moderate physical exercise (<5 h/week) reduced the 8-OH-dG level most significantly (P=0.0039) as compared to that of other people (non-exercise/hard exercise group), while hard working conditions (physical labor, day-night shift work) had an increasing effect. Although physical labor was not significantly correlated with the 8-OH-dG level in the multiple regression analysis of the nine selected variables (P=0.058) (Table IV), it seems to induce oxidative stress because it was third in the forward selection procedure. These data are compatible with the data from our previous rat experiments, in which the spontaneous exercise group showed significantly lower 8-OH-dG levels in DNA as compared to the forced exercise group.20 The sedentary control group showed an intermediate 8-OH-dG level. Oxygen consumption alone is not enough to explain these phenomena. Other factors, such as the induction of oxygen radical scavenger enzymes and repair enzymes, may be involved in the lower 8-OH-dG levels in the moderate exercise group. It is worth mentioning that epidemiological studies show that colon, lung, and breast cancer are reduced by physical exercise.21–23

We found that low meat intake (less than once/week) induces an increase of 8-OH-dG. This is a very unex-
pected result. First, very low consumption of meat may be associated with the serious modulation of other dietary factors, which might induce oxidative stress. Another possibility is that components in meat may be required to scavenge oxygen radicals or to repair DNA damage efficiently. We also confirmed the previous finding by Loft et al., that cigarette smoking is positively correlated with the 8-OH-dG level and that BMI is inversely correlated with it.12

It should be mentioned that the mean 8-OH-dG level of our analysis (4.12 µg/g creatinine) is similar to that determined by another group with the HPLC-ECD method (3.68–3.96 µg/g creatinine)9 and to that measured by a GC-MS-method (3.33–3.95 µg/g creatinine) using an isotope dilution technique,13 but is four times lower than that determined by an ELISA method (17.1 µg/g creatinine).13 The following factors may explain the discrepancy with the ELISA method: 1) the antibody used in the ELISA cross-reacts with other structurally related compounds; 2) the 8-OH-dG is excreted as further modified forms, or as a component of oligo-nucleotides in urine as a result of nucleotide excision repair.

In the present study, only limited lifestyle information was obtained with a single sheet questionnaire. Statistical analyses using more detailed information on diet, mental stress, status of hepatitis virus and H. pylori infection, and environmental air pollution will be required to identify the causes of oxidative DNA damage in humans. The genetic status of oxygen radical scavenger enzymes or repair enzymes may also influence the levels. In contrast to the analysis of 8-OH-dG in DNA, its analysis in urine is more reproducible because of the lack of artifact formation, and the inter-laboratory deviation seems to be low. Therefore, it should be easier to assess the effects of lifestyle factors and genotoxic environmental chemicals on cellular oxidative stress by analyzing 8-OH-dG in animal and human urine.

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