8-Hydroxyguanine in Urine and Serum as an Oxidative Stress Marker: Effects of Diabetes and Aging

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Abstract: 8-Hydroxydeoxyguanosine (8-OH-dG) is the most extensively analyzed oxidative stress marker. Recently, 8-hydroxyguanine (free base: 8-OH-Gua) has been recognized as an oxidative stress marker. To verify the usefulness of 8-OH-Gua, the 8-OH-dG and 8-OH-Gua levels in the urine and the 8-OH-Gua levels in the serum of type 2 diabetic model animals, db/db mice, were measured as oxidative stress markers by a column switching HPLC-system coupled to an electrochemical detector. The urinary 8-OH-Gua and 8-OH-dG levels in db/db mice (7-26 weeks old) were significantly higher than those in control (db/m+) mice. The 8-OH-Gua levels in the serum of the db/db mice were also about 2-fold higher than those in the control mice at 26 weeks of age. In addition, the urinary levels of 8-OH-dG and 8-OH-Gua increased with age (9-26 weeks). A significant positive correlation was obtained between the 8-OH-dG and 8-OH-Gua levels in urine. Although no difference was observed in the 8-OH-dG levels in the liver and kidney DNA between the diabetic and control mice, these results suggested that urinary 8-OH-dG and free base 8-OH-Gua in urine or serum may be good biomarkers of oxidative stress.

Keywords: diabetes, 8-hydroxydeoxyguanosine, 8-hydroxyguanine, oxidative stress, aging.

Introduction

Reactive oxygen species, formed in cells by various environmental agents and endogenous oxygen metabolism, damage DNA [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 [2–5], because 8-OH-dG induces mutations [6, 7]. In addition to 8-OH-dG, the free base, 8-hydroxyguanine (8-OH-Gua), in biological fluids may be a useful marker of oxidative stress in vivo [8–10]. The relationship between 8-OH-Gua and 8-OH-dG is as in Fig. 1. We have reported increased 8-OH-Gua levels in urine and serum after mice were exposed to ionizing radiation [11], and found that urinary 8-OH-Gua showed a better linear negative correlation with the species’ potential life spans (mice, rats, guinea pigs, cats, chimpanzees and humans) than urinary 8-OH-dG [12]. 8-OH-Gua may be generated by either base excision repair or guanine oxidation in a salvage pathway [4]. Therefore, 8-OH-Gua should also be assessed as an oxidative stress marker.

Some studies have suggested that diabetes increases oxidative stress, which plays a key role in the development of diabetic complications [13–15]. 8-OH-dG has
often been used as a biomarker of oxidative DNA damage in relation to diabetes mellitus [16–19]. However, many studies have measured urinary 8-OH-dG levels by enzyme-linked immunosorbent assay (ELISA) [20, 21], and there are frequent discrepancies among the results from various groups. These problems may arise from differences in the analytical methods. We previously proved that the 8-OH-dG antibody in the commercial ELISA kit reacts with not only 8-OH-dG but also with urea and 8-hydroxyguanosine (8-OH-G) [22]. Therefore, the 8-OH-dG levels determined with this kit, and especially the urinary 8-OH-dG levels, may be overestimated.

In this report, we accurately measured the 8-OH-dG and 8-OH-Gua levels in db/db mice (type 2 diabetes model) by a column switching HPLC-system coupled to an electrochemical detector (ECD), in order to assess the possible use of 8-OH-Gua as a biomarker of oxidative stress in a diabetes study. Additionally, as it has been reported that the amount of oxidative damage increases as an organism ages [23, 24], we also assessed the 8-OH-Gua changes that occur with aging.

**Materials and methods**

**Materials**

2-Acetamido-6-hydroxypurine (acetyl guanine) was purchased from Tokyo Kasei Kogyo Co., Ltd., Japan.

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**Fig. 1. Sources of 8-OH-Gua and 8-OH-dG.**

- dGTP: deoxyguanosine triphosphate
- 8-OH-dGTP: 8-hydroxydeoxyguanosine triphosphate
- 8-OH-Gua: 8-hydroxydeoxyguanine
- 8-OH-dG: 8-hydroxydeoxyguanosine

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**Animal experiments**

Five-week-old obese diabetic female BKS Cg +Lepr db/+Lepr db (db/db) mice (n = 10), a rodent model of type 2 diabetes, and non-diabetic BKS Cg +Lepr db/ m+ (db/m+) mice (n = 8) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The mice were fed a commercial diet lacking 8-OH-dG (Dyet no.110952; Dyets, Inc. Bethlehem, PA, USA) ad libitum and tap water. The mice were kept in a temperature-controlled room with a 12 hr light/dark cycle. For urine collection, the mice were housed in metabolic cages for 24 hr. The 24 hr urine output was collected and stored at −20°C until the assay. The mice were sacrificed at 26 weeks of age. The liver and kidneys were collected from each mouse for 8-OH-dG analysis, and were stored at −80°C. The whole blood was collected from the inferior vena cava under ether anesthesia, and was separated by centrifugation. The serum samples thus obtained were stored at −20°C until analysis. All procedures were performed according to the guidelines for the care and use of laboratory animals at the University of Occupational and Environmental Health, Japan. Blood glucose, body weight, and diet and water consumption were measured during the experiments.

**Analysis of 8-OH-dG in DNA**

The analysis of 8-OH-dG in DNA was performed as described previously [11], with a slight modification. Briefly, the nuclear DNA from the liver and kidney was isolated using a DNA Extraction WB Kit (Wako Pure Chemical Industries Ltd., Osaka, Japan), according to the manufacturer’s instructions. To avoid oxidative DNA artifacts, deferoxamine mesylate (Sigma Chemical Co., MO, USA) was added to the lysis solution for DNA extraction (final concentration, 1 mM). The isolated DNA was digested with nuclease P1 (Yamasa Corp., Choshi, Japan) and alkaline phosphatase (Roche Diagnosis GmbH, Mannheim, Germany). The digested DNA solution was filtered through a pretreatment filter (EKICRODISC, Acro LC3CR, Nihon Pall Ltd., Tokyo, Japan) and then stored at −80°C until analysis. The filtrate was injected into an HPLC column (Capcell Pak C18 MG II, 3 µm, 4.6 × 100 mm + 150 mm (series-connected), Shiseido Fine Chemicals, Tokyo, Japan) equipped with UV (UV-8020, Tosoh...
Co., Tokyo, Japan) and ECD (ECD-300, Eicom Co., Kyoto, Japan, applied voltage: 550 mV) detectors. The mobile phase was 10 mM NaH₂PO₄, containing 8% methanol and 0.13 mM Na₂EDTA, delivered at a flow rate of 0.7 ml/min. The column temperature was 35°C. The amount of 8-OH-dG in the DNA was determined by comparison to the authentic standard. The 8-OH-dG value in the DNA was calculated as the amount of 8-OH-dG per 10⁶ deoxyguanosine (dG).

Analysis of urinary 8-OH-dG

The urinary 8-OH-dG level was measured according to the method developed in our laboratory [25]. Briefly, the urine samples were defrosted at room temperature and mixed completely. A 60 µl aliquot of the urine was then mixed with the same volume of a dilution solution containing the ribonucleoside marker 8-hydroxyguanosine. This solution was incubated at 37°C for 40 min and then centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant was filtered through a pretreatment filter (EKICRODISC, Acro LC3CR, Nihon Pall Ltd., Tokyo, Japan). The filtrate (20 µl) was fractionated on an anion exchange column (65°C, 2% acetonitrile in 0.3 mM sulfuric acid). The 8-OH-dG fraction was collected according to the peak position of the marker 8-hydroxyguanosine, detected by a UV detector (245 nm), and was automatically injected into the reverse phase column (50°C, 2% methanol in 10 mM phosphate buffer, pH 6.0). The 8-OH-dG level was determined by using an EC detector (Coulochem III EC detector, ESA, Chelmsford, MA, USA) with a guard cell and an analytical cell (applied voltages: guard cell, 400 mV; E1, 150 mV; E2, 350 mV).

Analysis of urinary 8-OH-Gua

The urinary 8-OH-Gua levels were measured according to the method of Kawai et al. [11], with slight modifications. Briefly, frozen urine samples were defrosted at room temperature and mixed. A 50 µl aliquot of urine was then mixed with the same volume of a diluent containing acetylguanine (AcG, 70 µg/ml), 4% acetonitrile, 130 mM NaOAc, pH 4.5 and 0.6 mM H₂SO₄, and incubated at 37°C for 40 min. The diluted samples were centrifuged at 13,000 rpm for 5 min. After centrifugation, a 70 µl portion of the supernatant was reserved for the 8-OH-Gua analyses. A 20 µl aliquot of the prepared solution was fractionated on an anion exchange column. The 8-OH-Gua fraction was collected based on the peak position of the added marker AcG, detected at 245 nm, and was automatically injected into the HPLC-2 column. An EC detector (ECD-300, Eicom Co., Kyoto, Japan, applied voltage: 600 mV) was connected to the HPLC-2 for the 8-OH-Gua analyses. The conditions for the HPLC-2 were as follows: mobile phase: 10 mM NaH₂PO₄ containing 2% methanol and 0.13 mM Na₂EDTA; flow rate: 0.7 ml/min; column temperature: 33°C.

Analysis of 8-OH-Gua in serum

The 8-OH-Gua in the serum was determined according to the previously described method [11]. Briefly, a 50 µl serum sample was digested with 10 µl of 20 mg/ml proteinase K (Sigma Chemical Co., MO, USA) at 37°C for 1 hr. Before analysis, the digested serum solution was mixed with 60 µl of a dilution solution (the same solution used for the urinary 8-OH-Gua dilution). After centrifugation at 13,000 rpm for 5 min, a 70 µl aliquot of the supernatant was used to analyze 8-OH-Gua. The analysis conditions were the same as those for the urinary 8-OH-Gua.

Statistical analysis

Statistical analyses were performed using the Student t-test and the Steel test. Statistical significance was assessed as P < 0.05. In order to correct the urine concentration, urinary levels of 8-OH-dG and 8-OH-Gua were calculated as the each amount per creatinine levels.

Results

Characteristics of the animals

The characteristics of the animals are shown in Table 1. The body weight, diet and water consumption, and blood glucose level in the db/db mice were significantly higher than those in the db/m+ mice.

Analysis of 8-OH-dG in urine and nuclear DNA

The urinary 8-OH-dG levels in the db/db mice were significantly higher than those in the db/m+ mice at all ages, as shown in Fig. 2. The ratios of urinary 8-OH-
dG levels in the db/db mice to those in the db/m+ mice at each age ranged from 2.1 to 3.8. Furthermore, significant increases in the 8-OH-dG levels were detected in the db/db (26 weeks) and db/m+ (13 and 26 weeks) mice with aging.

On the other hand, no difference in the 8-OH-dG levels was observed in the liver and kidney nuclear DNA between the db/db and db/m+ mice (data not shown).

### Analysis of 8-OH-Gua in urine and serum

The urinary 8-OH-Gua levels in the db/db mice were significantly higher than those in the db/m+ mice (Fig. 3). The value of 8-OH-Gua in the db/db mice was about 2.6-fold higher than that in the db/m+ mice at 7 weeks of age, and 4-fold higher at 26 weeks. Significant increases in the 8-OH-Gua levels were observed in both the db/db (26 weeks) and db/m+ (13 and 26 weeks) mice with aging. In addition, a highly

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### Table 1. The characteristics of the animals

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mouse</th>
<th>Age in weeks</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>db/m+</td>
<td>20.49 ± 1.17</td>
</tr>
<tr>
<td></td>
<td>db/db</td>
<td>33.61 ± 1.43**</td>
</tr>
<tr>
<td>Diet consumption (g / day)</td>
<td>db/m+</td>
<td>3.23 ± 0.65</td>
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<tr>
<td></td>
<td>db/db</td>
<td>5.44 ± 0.46**</td>
</tr>
<tr>
<td>Water consumption (ml / day)</td>
<td>db/m+</td>
<td>1.64 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>db/db</td>
<td>5.79 ± 2.02**</td>
</tr>
<tr>
<td>Blood glucose (mg / dl)</td>
<td>db/m+</td>
<td>123.88 ± 12.12</td>
</tr>
<tr>
<td></td>
<td>db/db</td>
<td>327.60 ± 101.52**</td>
</tr>
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</table>

**: P < 0.01 (significant difference from db/m+ mice by Student t-test)

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**Fig. 2. Urinary 8-OH-dG levels in db/db and db/m+ mice.** Columns represent mean ± SD. N = 6 to 10. *: P < 0.05 (significant difference from db/m+ mice by Student t-test). #: P < 0.05, #: #: P < 0.01 (significant difference from the level at 7 weeks in each strain by Steel test). [Graph image]

**Fig. 3. Urinary 8-OH-Gua levels in db/db and db/m+ mice.** Each value represents mean ± SD. N = 5 to 10. *: P < 0.05, **: P < 0.01 (significant difference from db/m+ mice by Student t-test). #: P < 0.05, #: #: P < 0.01 (significant difference from the level at 7 weeks in each strain by Steel test). [Graph image]
significant positive correlation was obtained between the 8-OH-dG and 8-OH-Gua levels ($r^2 = 0.7242, P < 0.001$) (Fig. 4).

The 8-OH-Gua levels in serum at 26 weeks also revealed a remarkable difference between the db/db mice and db/m+ mice (Fig. 5), being about 2-fold higher in the db/db mice than in the db/m+ mice. Positive correlations were also obtained between the serum 8-OH-Gua and urinary 8-OH-Gua levels ($r^2 = 0.703, P < 0.001$), and between the serum 8-OH-Gua and urinary 8-OH-dG levels ($r^2 = 0.662, P < 0.001$) (Fig. 6).

**Discussion**

8-OH-dG is often used as a biomarker for oxidative stress in diabetes studies[14, 26, 27]. Experimental and clinical studies have suggested that increased oxidative stress is associated with the diabetic condition [14, 26, 27]. In the present study, to avoid the possible over-estimation of 8-OH-dG levels by an ELISA or
immunohistochemical analysis, an HPLC-ECD method was performed.

Our present results were in agreement with previous studies that suggested that the urinary 8-OH-dG levels in diabetic patients or model animals were significantly higher than those in a non-diabetic group [21, 28–30]. We also found an increase in urinary 8-OH-dG levels with aging (9~26 weeks) in db/db and db/m+ mice. It is reasonable to speculate that the urinary 8-OH-dG levels of db/db mice are higher than those of db/m+ mice at all ages, due to oxidative stress, and the lifespan of db/db mice (about 10 months) is actually shorter than that of db/m+ mice (about two years).

In our studies, the 8-OH-dG levels in the liver and kidney nuclear DNA of db/db mice were not significantly different from those of db/m+ mice, although Fujii et al. [29] reported an increased amount of 8-OH-dG in the kidneys of 14-week old db/db mice, based on an immunostaining analysis. Park et al. reported increased 8-OH-dG levels in the liver and kidney DNA of streptozotocin-induced diabetic rats [31]. Our results indicated that oxidative damage is not induced in liver and kidney nuclear DNA. This is compatible with the conclusion reported by Kakimoto et al. [32], who found an accumulation of 8-OH-dG only in the mitochondrial DNA (mtDNA), not in the nuclear DNA, of streptozotocin-induced diabetic rat kidneys. The mtDNA might be exposed to higher oxidative stress as compared with the nuclear DNA, because the mitochondrial respiratory chain is the source of a continuous flux of oxygen radicals, and mtDNA lacks both protection by histones and an efficient repair system for DNA damage [33].

In addition to 8-OH-dG, the free base 8-OH-Gua is another abundant type of guanine damage generated by reactive oxygen species (ROS) [34], and was proved to be a useful marker of oxidative stress in vivo [11]. However, few reports have analyzed the serum and plasma 8-OH-Gua levels in diabetes models [31, 35]. Therefore, in this study, the 8-OH-Gua levels in urine and serum were determined (Figs 2, 4). We found that the serum 8-OH-Gua levels in db/db mice were about 2-fold higher than those in db/m+ mice (db/m+: 0.41 ± 0.15 ng/mL; db/db: 0.84 ± 0.28 ng/mL). Park et al. [31] reported that the plasma 8-OH-Gua levels in streptozotocin-induced diabetic rats were three times higher than those in control rats (control: 3.3 ± 2.7 pmol/mL; diabetes: 10.4 ± 2.3 pmol/mL). These results indicated that serum or plasma 8-OH-Gua could be a useful biomarker of oxidative stress in diabetic subjects.

This is the first report to measure the urinary 8-OH-Gua levels accurately in a diabetic animal model. The urinary 8-OH-Gua levels in db/db mice were significantly higher (7-weeks: 2.6-fold; 26-weeks: 4-fold) than those in db/m+ mice. This finding suggests that urinary 8-OH-Gua could also be a useful biomarker of oxidative stress in diabetes studies. In addition, the amount of 8-OH-Gua in the serum and urine were much higher than that of 8-OH-dG. This is advantageous for accurate measuring. Actually, the urinary 8-OH-Gua levels could detect in all samples even the urinary 8-OH-dG levels of some db/db mouse were lower than the minimum detection limit.

In conclusion, our results showed that the 8-OH-Gua levels in urine and serum are significantly increased in a type 2 diabetes mouse model, and this trend is similar to that of the urinary 8-OH-dG. Furthermore, the levels of both 8-OH-Gua and 8-OH-dG increased with advancing age. Therefore, 8-OH-Gua, in addition to 8-OH-dG, could be a useful biomarker of oxidative stress in diabetes and aging studies.

Acknowledgement

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酸化ストレスマーカーとしての尿中ならびに血清中の8-ヒドロキシグアニン：糖尿病ならびに加齢の影響

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要 旨：8-ヒドロキシデオキシグアノシン(8-OH-dG)は、酸化ストレスマーカーとしてもっとも広く分析されている。これに加え、フリーの塩基である8-ヒドロキシグアニン(8-OH-Gua)の酸化ストレスマーカーとしての有用性が最近注目され始めた。本研究では、その有用性を示す目的で、尿中ならびに血清中の8-OH-Guaレベルを、酸化ストレスの亢進が予測される動物(2型糖尿病モデルマウスdb/db)を用いて測定し、代表的な酸化ストレスマークーアである尿中8-OH-dGの測定結果と比較検討した。測定は、感度・精度に優れたHPLC-ECD法で行った。db/dbマウスの尿中8-OH-Guaならびに8-OH-dG値は、非糖尿病マウス(db/m+)に比べて有意に高い値を示した。（例えば26週齢では、db/db: 8-OH-Gua 1.84 ± 1.00 mg/mg creatinine, 8-OH-dG 64.72 ± 27.69 ng/mg creatinine, db/m+: 8-OH-Gua 0.37 ± 0.08 mg/mg creatinine, 8-OH-dG 17.06 ± 1.11 ng/mg creatinine)。また、それぞれの値は、動物の週齢（13～26週）に伴って増加する傾向が見られ、加齢による酸化ストレスの亢進が観察された。さらに尿中の8-OH-Gua値は、尿中8-OH-dG値と良い相関を示した。8-OH-Guaは血清（26週齢、db/m+: 0.41 ± 0.15 ng/ml; db/db: 0.84 ± 0.28 ng/ml）でも測定することが可能で、その値は尿中の値と良い相関関係にあった。糖尿病、加齢に伴う酸化ストレスを感度良く測定できたことから、尿中ならびに血清中の8-OH-Guaは、酸化ストレスのバイオマーカーとして利用が期待される。

キーワード：8-ヒドロキシグアニン、8-ヒドロキシデオキシグアノシン、糖尿病、加齢、酸化ストレス。