Review
Analysis of 8-OH-dG and 8-OH-Gua as Biomarkers of Oxidative Stress

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Reactive oxygen species (ROS) are generated by environmental factors, such as ionizing radiation and chemical carcinogens, and also by endogenous processes, including energy metabolism in mitochondria. In 1984, Kasai and colleagues first reported the detection of 8-hydroxydeoxyguanosine (8-OH-dG), a type of oxidative damage in DNA formed by Fenton-type reagents and X-irradiation in vitro. Further studies of the mechanisms of 8-OH-dG formation by various carcinogens suggested that it is generated by a wide variety of agents with different mechanisms of action. 8-OH-dG is biologically significant, since it induces mutations and has specific repair systems. 8-OH-dG is one of the major forms of oxidative DNA damage, and it has been well studied because it is relatively easy to detect by using a high performance liquid chromatography-electrochemical detector (HPLC-ECD) system. In this review, we summarize the methods to analyze 8-OH-dG in cellular DNA and urine, and the free base, 8-hydroxyguanine (8-OH-Gua), in urine and serum.

Key words: reactive oxygen species, 8-hydroxydeoxyguanosine, 8-hydroxyguanine, oxidative damage, biomarker

Introduction

The C-8 hydroxy modification of guanine was first detected when mutagens in heated glucose, used as a model of cooked food, were trapped as adducts with a guanosine derivative, isopropylideneguanosine (IPG) (1). The mutagens in heated carbohydrates that cause this guanine modification were later found to be reductive acid derivatives that produce ROS (2). This unexpected discovery triggered studies on the mechanism of 8-hydroxyguanine (8-OH-Gua) formation by ROS in DNA, as well as its mutagenic properties and repair enzymes. Our discovery also supported the hypothesis by Ames, stating that ROS-forming mutagens are important dietary mutagens, and that the intake of antioxidants that scavenge ROS is a good strategy for cancer prevention (3). Recently, the effects of ROS on the pathogenesis of various diseases, including cancer, diabetes and heart disease, have been widely studied. However, because ROS themselves have rather short half-lives, it is difficult to measure them directly. Therefore, the accurate measurement of the DNA oxidation product, 8-OH-dG, is important to estimate the risk of these diseases.

Since the first report that 8-hydroxydeoxyguanosine (8-OH-dG) can be detected by HPLC-ECD with high sensitivity (4), many improvements have been made in DNA isolation and digestion techniques to prevent the artificial formation of 8-OH-dG, and recently, a reliable technique for 8-OH-dG analysis from cellular DNA was established. We have reported that many ROS forming mutagens and carcinogens induce the formation of 8-OH-dG in target organ DNA. To investigate the role of oxidative stress in carcinogenesis caused by human lifestyle choices, it is useful to measure the 8-OH-dG levels in human peripheral leukocytes or urine. Kasai developed an automated HPLC-ECD system to analyze urinary 8-OH-dG with high accuracy (5). Several lifestyle factors, such as smoking, drinking, exercise, working conditions and stress, correlated with the 8-OH-dG levels.

As sources of urinary 8-OH-dG, two pathways are possible (Fig. 1). First, it is produced from 8-OH-dGTP formed in the nucleotide pool, followed by the removal of pyrophosphate by the MTH1 nucleotide sanitization enzyme (6), and the subsequent digestion of 8-OH-dGMP by a 5′(3′)-nucleotidase. Second, 8-OH-dG is produced by nucleotide excision repair (NER)-type mechanisms. As an example, Bessho et al. reported an 8-OH-dG repair mechanism involving an endonuclease that generates 3′,5′-8-OH-dGDP (7), which may be further hydrolyzed to 8-OH-dG by the nucleotidase. Daninov et al. reported that 25% of the 8-OH-dG in DNA is repaired by the long patch NER pathway in mammalian cells (8). Takeuchi and his collaborators

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also suggested the presence of an endonuclease/NER-type repair system in rat liver homogenates that generates 8-OH-dG from 8-OH-dG-containing DNA (9). On the other hand, as sources of the free base, 8-OH-Gua, in urine and serum, the base excision-type repair of oxidized DNA, and the oxidation of guanine formed by the hydrolytic degradation of DNA, RNA and the nucleotide are possible (Fig. 2). However, further studies are required to clarify these hypotheses.

During the last two decades, many reports about 8-OH-dG have been published (more than 3000 reports). In parallel, publications on urinary 8-OH-dG (about 500 reports) and the inhibition of 8-OH-dG by antioxidants (about 800 reports) also increased. In this review, we summarize the methods to analyze 8-OH-dG in cellular DNA and urine, and the free base, 8-OH-Gua, in urine and serum used in our laboratory. We will show how these oxidative stress markers are useful for studies on oxidative stress by describing our recent data, such as X-irradiated mouse experiments.

**Determination of 8-OH-dG in Cellular DNA**

In the recently improved method of 8-OH-dG analysis, cells are homogenized in the presence of desferal, an iron chelating agent, and DNA is isolated by the modified chaotropic NaI technique (10,11). With these improvements, the background level of 8-OH-dG was reduced, and the range of estimates of 8-OH-dG has fallen and narrowed considerably. The method currently used to determine 8-OH-dG in cellular DNA in our laboratory is described below.

Cellular DNA is isolated using a DNA extractor WB kit containing NaI (Wako, Osaka, Japan). Desferal (deferoxamine mesylate) is added to the lysis solution (1 mM) to prevent DNA oxidation. The isolated DNA is digested with 8 units of nuclease P1, in a 100 μL solution containing 1 mM EDTA and 20 mM sodium acetate (pH 4.5), and then is treated with alkaline phosphatase (2 units) in 250 mM Tris-HCl (pH 8.0) buffer. This DNA digest is then filtered with an Ultrafree-Probind filter, and a 70 μL aliquot of the sample is injected into an HPLC column (5 μm, 4.6 × 250 mm; temperature, 32°C; flow rate, 1.0 mL/min) equipped with an
Table 1. Formation of 8-OH-dG and repair enzyme response after treatment with ROS-forming agents

<table>
<thead>
<tr>
<th>Animal organs</th>
<th>8-OH-Gua level in DNA</th>
<th>Repair activity (OGG1 type)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-Rays</td>
<td>↑</td>
<td>↓</td>
<td>[13]</td>
</tr>
<tr>
<td>Arsenite</td>
<td>↑</td>
<td>↓</td>
<td>[14]</td>
</tr>
<tr>
<td>Animal organs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromium (VI)</td>
<td>↑</td>
<td>↓</td>
<td>[15]</td>
</tr>
<tr>
<td>Cadmium (GSH depletion)</td>
<td>↑</td>
<td>↓</td>
<td>[16]</td>
</tr>
<tr>
<td>Diesel exhaust particles</td>
<td>↑</td>
<td>↓</td>
<td>[17]</td>
</tr>
<tr>
<td>Ethanol (Nutrition deficient)</td>
<td>↑</td>
<td>↑</td>
<td>[18]</td>
</tr>
<tr>
<td>3′-Me-4-DAB</td>
<td>↑</td>
<td>↑</td>
<td>[19]</td>
</tr>
<tr>
<td>Fe-NTA</td>
<td>↑</td>
<td>↑</td>
<td>[20]</td>
</tr>
<tr>
<td>Asbestos</td>
<td>↑</td>
<td>↑</td>
<td>[21]</td>
</tr>
</tbody>
</table>

Cultured cells repair activity. These potent carcinogens, Cd, Cr and DEP, may enhance the accumulation of 8-OH-dG by impairing the repair activity. Enhancement of 8-OH-dG by impaired repair activity may result in the development of cancer. The 8-OH-dG repair activity was impaired in the target organs, esophagus, liver, kidney, and lung. Cigarette smoking also increased the levels of both 8-OH-dG and its repair activity in human leukocytes. On the other hand, with the administration of cadmium (Cd) to rats, under conditions of glutathione depletion, the 8-OH-dG repair activity was impaired in the target organ, testis, while the 8-OH-dG levels in the DNA were increased. When diesel exhaust particles (DEP) were intratracheally administered to rats, or a hexavalent chromium (Cr) mist was administered to rats by inhalation, again the repair activity was decreased in the lungs, while the 8-OH-dG levels in the DNA were increased. These potent carcinogens, Cd, Cr and DEP, may enhance the accumulation of 8-OH-dG by impairing the repair activity.

Method to Analyze Urinary 8-OH-dG

Since Ames and his collaborators first reported the presence of 8-OH-dG in human, rat and mouse urine (22), several groups have reported its analysis by different methods. These include 1) the high performance liquid chromatography (HPLC) method using a reverse phase column coupled to an electrochemical detector (ECD) after pre-purification (23), 2) the gas-chromatography mass spectrometry (GC-MS) method (24), 3) the HPLC-electrospray tandem mass spectrometry (LC-MS-MS) method (25), and 4) the enzyme-linked immunosorbent assay (ELISA) method (26). The urinary 8-OH-dG levels measured by the ELISA method were at least two times higher than those obtained by the HPLC-ECD method (27), and also the reproducibility of the analyses was very low (28).

We developed an automated HPLC-ECD system to analyze urinary 8-OH-dG. Our method is unique in that, 1) the urine is first fractionated by anion exchange chromatography (polystyrene-type resin with a quaternary ammonium group, sulfate form) prior to analysis by reverse phase chromatography; and 2) the 8-OH-dG fraction in the first HPLC is precisely and automatically collected, based on the added ribonucleoside 8-hydroxyguanosine (8-OH-G) marker peak, which elutes a few minutes earlier. Up to 700 human urine samples can be continuously analyzed with high accuracy within a month.

Apparatus and 8-OH-dG analysis: The urinary 8-OH-dG level was determined using the apparatus, in which three pumps, the sampling injector, two valves, the guard column for HPLC-1, the main HPLC-1 column, the UV detector, the HPLC-2 column, and the EC detector were connected. The guard and main columns for HPLC-1 were set in a column oven at 65 °C, and the HPLC-2 column was set in a column oven at 40°C. A 20 μL aliquot of the diluted urine sample was injected into HPLC-1 (MCI GEL CA08F, 7 μm, 1.5 × 120 mm, solvent A, 50 μL/min) from the sampling injector, via the guard column, and the chromatogram was recorded by a UV detector (245 nm). In this method, the 8-OH-dG fraction was unequivocally and precisely collected, depending upon the relative elution position from the peak of the added marker, 8-OH-G, and was automatically injected into the HPLC-2 column coupled with the ECD (Fig. 3). By monitoring two ECD channels with different applied voltages, for example, 170 and 300 mV, the 8-OH-dG peaks appear with a specific ratio of peak heights, as shown in Fig. 4. Under these standard conditions, one sample was analyzed every 30–40 min. Usually, the analysis of 30–35 samples is possible per day.

Creatinine (Cre) is frequently used as an internal standard for normalizing urinary 8-OH-dG. We attempted
to analyze both 8-OH-dG and Cre simultaneously in the HPLC-1 step with our method. When we chose 245 nm for monitoring in HPLC-1, and used a thinner UV cell (0.2 mm light path), we successfully detected the Cre peak, as shown in Fig. 3 (29). It was also possible to measure 7-methylguanine (7-MG), a DNA methylation product in urine, in addition to Cre (30).

This convenient method for the simultaneous measurement of 8-OH-dG, Cre and 7-MG from the same urine sample may be widely used in the future for assessing the risk of environmental agents, and for evaluating the risk of oxygen radical-related diseases.

8-OH-dG and 8-OH-Gua as Useful Biomarkers of Oxidative Stress

Mouse experiments: To determine whether the 8-OH-dG or 8-OH-Gua levels in biological fluids or cellular DNA reflect in vivo oxidative stress, a typical oxygen radical forming agent, X-rays, was tested for the induction of these oxidative stress markers (12). After mice were irradiated with 2–30 Gy X-rays at a dose rate of 0.5 Gy/min, urinary 8-OH-dG and 8-OH-Gua, serum 8-OH-Gua, and 8-OH-dG in the liver DNA were measured. Among these markers, the urinary 8-OH-dG collected during the 24 h following irradiation was most dramatically increased by low dose X-irradiation (Fig. 5). After irradiating with 2 Gy X-rays, the excretion of 8-OH-dG increased 4-fold. Immediately after irradia-
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Fig. 5. Increase of urinary 8-OH-dG after X-ray irradiation of mice. The p value was calculated for the Spearman’s rank correlation.

Fig. 6. Increase of 8-OH-dG in mouse liver DNA after X-ray irradiation. The p value was calculated for the Spearman’s rank correlation.

Fig. 7. Analysis of 8-OH-Gua in mouse serum.

Fig. 8. Increase of mouse serum 8-OH-Gua after X-ray irradiation. The p value was calculated for the Spearman’s rank correlation.

Fig. 9. Increase of urinary 8-OH-dG after X-ray irradiation of mice. The p value was calculated for the Spearman’s rank correlation.

Fig. 10. Increase of urinary 8-OH-Gua after X-ray irradiation. The p value was calculated for the Spearman’s rank correlation.

To clarify the effect of diet on oxidative stress, urinary 8-OH-dG was analyzed in mice by HPLC-ECD. As a model of a vitamin-deficient diet, an autoclaved diet was fed to mice. Autoclaving the diet destroys 50% of vitamins A and C, which are known as cancer preventive vitamins. In this experiment, the urinary 8-OH-dG levels were increased to 1.2-fold and 1.4-fold after one month and two months, respectively (Fig. 9) (31). When mice were given a commercial sweet beverage instead of water for two weeks, the urinary 8-OH-dG was increased to 1.4-fold (Fig. 10). Some epidemiological studies have shown that the excess intake of sugar is cor-
related with the induction of colon (32), pancreatic (33,34) and breast (35) cancer. These results suggested that the prolonged intake of a vitamin-deficient diet or a sweet beverage will destroy the normal nutrient balance, induce oxidative DNA damage, and increase the risk of cancer.

**Human studies:** One of the mechanisms of the genotoxicity of asbestos fibers appears to be the generation of ROS, either from its surface, by reactions involving catalytic iron, or from its phagocytosis by frustrated phagocytes. We demonstrated a positive correlation between the 8-OH-dG levels in leukocyte DNA and the grades of asbestosis at a Chinese asbestos plant (36). A German group also conducted a study on 496 asbestos-exposed workers, in order to determine whether asbestos induces the formation of 8-OH-dG in white blood cells (37). The data from this study showed a 1.7–2-fold increase in 8-OH-dG due to asbestos exposure (p<0.001). These data support the hypothesis that asbestos fibers damage cells through an oxidative mechanism. In addition, preventive and therapeutic approaches using antioxidants may be possible.

To clarify the relationship between lifestyles and oxidative DNA damage, the urine samples of 372 employees of a company were analyzed (38). Alcohol drinking, cigarette smoking, average working hours and serum cortisol, a stress hormone, showed positive correlations with the 8-OH-dG level, while BMI, consumption of soybean products, rice and light-colored vegetables showed negative correlations.

An increased risk of some forms of cancer, including lung cancer, among lean individuals has been reported; however, there is limited biological evidence supporting this relation. The urinary 8-OH-dG levels of 177 healthy Japanese workers who participated in a lifestyle intervention study were analyzed. A clear inverse association was found between body mass index (BMI) and 8-OH-dG levels among smokers [Pearson correlation coefficient (r) = −0.48] (39). In contrast, no apparent relation was observed between BMI and 8-OH-dG levels (r = −0.12) among nonsmokers. Therefore, leanness may enhance the oxidative DNA damage induced by smoking, and 8-OH-dG may be a useful marker of host susceptibility to smoking-related cancers.

Patients with cancer (urine), chronic hepatitis (urine), diabetes (urine, leukocyte DNA), heart disease (leukocyte DNA), Alzheimer’s disease (urine), Parkinson’s disease (urine), as well as atopic dermatitis (urine), showed higher levels of 8-OH-dG (40). In contrast, the consumption of vitamins E and C, β-carotene, crucu- mine, green tea, red wine, and tomato sauce was correlated with a reduction in the amount of 8-OH-dG in urine or cellular DNA (40). Therefore, 8-OH-dG is a useful marker for monitoring the cellular oxidative stress involved in the induction of cancer and lifestyle-related diseases and their prevention by antioxidants.

In conclusion, oxidative stress is believed to increase the risk of lifestyle-related diseases, such as cancer and heart disease. Furthermore, since we use oxygen molecules as a source of energy, we will never be able to avoid oxidative damage. Therefore, 8-OH-dG studies are undoubtedly useful to identify ways to prevent cancer and ROS-related diseases. For this purpose, accurate measurements are essential. Here, we have summarized our improved analysis methods of 8-OH-dG and its application.

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