

Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair

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8-Hydroxydeoxyguanosine (8-OH-dG) was detected in DNA isolated from HeLa cells after the cells in tissue culture had been irradiated with X-rays and from the liver of mice after the whole animals had been irradiated with γ-rays. The amounts of 8-OH-dG in DNA after *in vivo* irradiation were three orders of magnitude lower than those after *in vitro* irradiation (0.008–0.032 8-OH-dG residue/10⁵ dG/krad). The 8-OH-dG produced in liver DNA by irradiation of mice decreased with time, suggesting the presence of a repair enzyme(s) acting on 8-OH-dG in mouse liver. Treatment of *Salmonella typhimurium* cells with hydrogen peroxide also caused increase in the 8-OH-dG content. These results indicate that 8-OH-dG is formed *in vivo* in cellular DNA on treatment with various oxygen radical-producing agents and that it is repairable.

Introduction

We previously found that the C-8 position of deoxyguanosine residues in DNA is hydroxylated to produce 8-hydroxydeoxyguanosine (8-OH-dG*) *in vitro* by various oxygen radical-producing agents, such as reducing agents (1), X-rays (2), asbestos plus hydrogen peroxide (H₂O₂) (3), and polyphenol with H₂O₂ and ferric ion (4). Since many mutagens, tumor promoters and carcinogens are known to generate oxygen radicals (5), and generation of oxygen radicals *in vivo* is thought to be relevant to carcinogenesis (6), 8-OHdG formation in DNA may be related to tumorigenesis. The question arises as to whether 8-OH-dG is formed in cellular DNA *in vivo* when intact cells are treated with oxygen radical-producing agents. In the present study we demonstrate that 8-OH-dG is in fact produced in cellular DNA *in vivo* by ionizing radiation or H₂O₂.

Materials and methods

Irradiation of HeLa cells and whole bodies of mice

HeLa cells (1 × 10⁶ cells) were cultured in Eagle's minimum essential medium supplemented with 5% calf serum, collected and suspended in 10 ml of Dulbecco's phosphate buffered saline (PBS). The cell suspension was divided into three pans: one as a control and the other two for irradiation. Cells were irradiated with 250 kVp X-rays generated by a Maxitron 300 (General Electric Co.) at a dose rate of 390 rad/min at 4°C with a 2 mm Cu filter. After irradiation, the cells were collected by centrifugation and quickly frozen at -80°C until isolation of DNA.

Female mice (C3H/He) were irradiated with γ-rays from a ⁶⁰Co source (1000 Ci, Toshiba Electric Co., Tokyo) at a dose rate of 130 rad/min and a total dose of 60 krad or 173 krad. In repair experiments, mice were irradiated with γ-rays from a ⁶⁰Co Cammacell (12000 Ci, Atomic Energy of Canada) at a dose rate of 14 krad/min. After irradiation, the mice were killed by cervical dislocation and their livers were quickly isolated and frozen at -80°C.

*Abbreviations: 8-OH-dG, 8-hydroxydeoxyguanosine; dG, deoxyguanosine; dT, thymidine; EC, electrochemical.

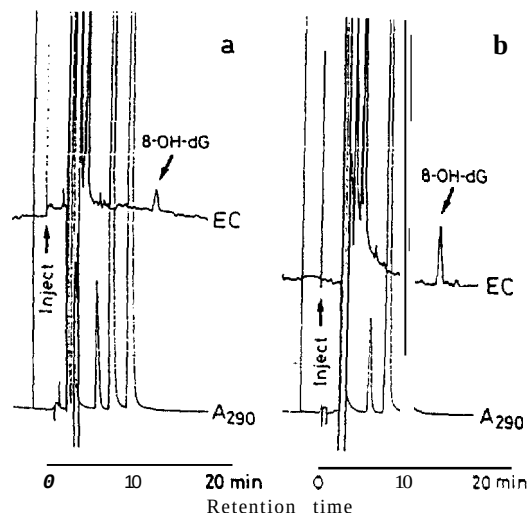


Fig. 1. Detection of 8-OH-dG with the h.p.l.c. - EC system. The detector response of EC and A₂₉₀ were recorded. (a) Control mouse liver DNA; (b) liver DNA from a mouse irradiated with 173 krad of γ-rays.

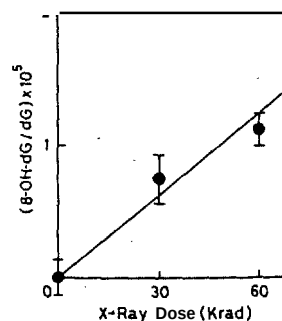


Fig. 2. Formation of 8-OH-dG in DNA of HeLa cells by X-ray irradiation. 8-OH-dG level in control DNA was subtracted. Points and bars represent mean values and standard deviations for three independent analyses.

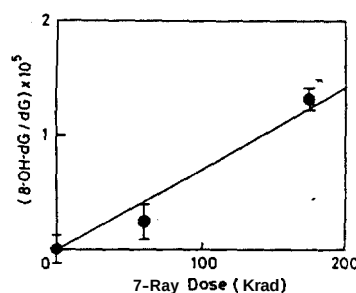
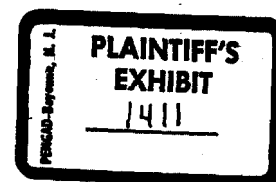


Fig. 3. Formation of 8-OHdG in mouse liver DNA on whole body irradiation with γ-rays.



Treatment of Salmonella typhimurium cells with H₂O₂

Salmonella typhimurium strain TA100 was grown in 1 l of nutrient broth (Difco) medium. A sample of 2 g of wet cells collected by centrifugation was suspended in 1 l of 100 mM sodium phosphate buffer (pH 7.4) and divided into four pans. One part was used as a control without treatment, and the other three were treated with different concentrations of H₂O₂ as specified at 4°C for 30 min. The cells were collected by centrifugation, suspended in 40 ml of the above buffer, and collected again by centrifugation. This washing procedure was repeated twice more and then the cells were frozen at -80°C.

Analysis of 8-OH-dG with an h.p.l.c.-electrochemical detector system

DNA was isolated from cells or tissues by Marmur's method (8), except that cells were lysed by 2% sodium dodecyl sulfate at 37°C for 30 min. Portions of DNA samples (4–5 A₂₆₀ units) were dissolved in 200 µl of 20 mM sodium acetate buffer (pH 4.8), digested with 20 µg of nuclease P1 at 37°C for 30 min, and then treated with 1.3 units of *E. coli* alkaline phosphomonoesterase in 0.1 M Tris-HCl buffer (pH 7.5) for 1 h.

The resulting deoxynucleoside mixture was injected into an h.p.l.c. apparatus coupled with an electrochemical (EC) detector (7): apparatus, Toyo Soda HLC-803D; column, Beckman Ultrasphere ODS (0.46 × 25 cm); eluent, 10% aqueous methanol containing 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NaOH, 10 mM acetic acid; flow rate, 1 ml/min; u.v. detector, Toyo Soda WV-8, 290 nm; EC detector, Toyo Soda EC-8000, 600 mV (oxidation). The molar ratio of 8-OH-dG to deoxyguanosine (dG) in each DNA sample was determined based on the peak height of authentic 8-OH-dG with the EC detector and the u.v. absorbance at A₂₉₀ of dG. On prolonged incubation of the DNA sample with *E. coli* alkaline phosphomonoesterase under the conditions described above, the content of 8-OH-dG increased at the rate of 0.3 ± 0.1 residue/10⁵ dG/h. Therefore this blank value for 8-OH-dG was subtracted from the observed data. In Figures 2–5, background 8-OHdC level in control DNA (0.6–1.4 residue/10⁵ dG) was subtracted.

Results

HeLa cells and whole bodies of mice were irradiated with different doses of X-rays and γ-rays, respectively. DNA was then isolated from HeLa cells and the livers of the mice, and the quantity of 8-OH-dG was determined as described in Materials and

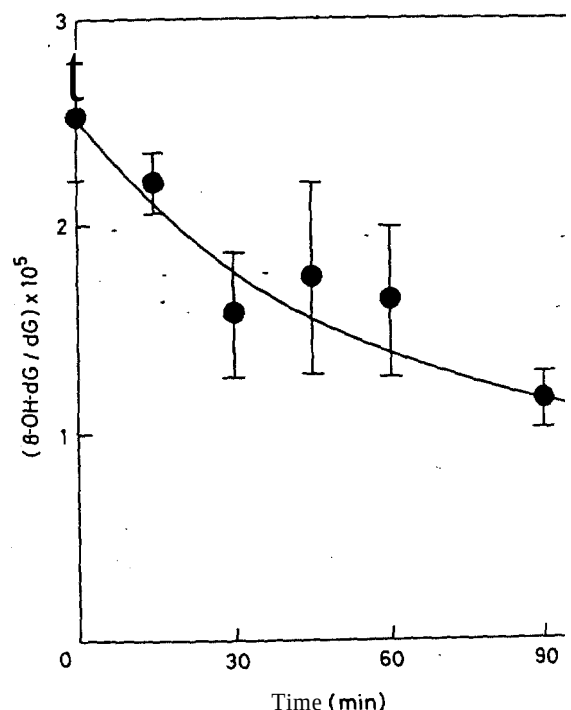


Fig. 4. Repair of 8-OHdC residues in liver DNA after whole body irradiation of mice with γ-rays. Mice were irradiated with 80 krad of γ-rays (14 krad/min). Livers were isolated at various times after irradiation and their DNA was analyzed as described in the experimental section.

methods. Typical chromatographic profiles of 8-OH-dG found in liver DNA of control mice and irradiated mice are shown in Figure 1. The irradiated DNA sample clearly contained more 8-OH-dG, but it is noteworthy that control liver also contained some 8-OH-dG. As shown in Figure 2 and 3, the content of 8-OH-dG increased with increase in the dose of ionizing radiation. The formation of 8-OH-dG in cellular DNA on radiation was estimated to be 0.008–0.018 residue/10⁵ dG/krad.

To determine whether 8-OH-dG in DNA is repaired *in vivo*, mice were killed at various times after irradiation with γ-rays (80 krad), and their liver DNA was analyzed. The 8-OHdG content in samples obtained 90 min after irradiation was considerably less than that in those obtained immediately after irradiation (Figure 4). This result suggests that mouse liver cells have a repair enzyme(s) for 8-OH-dG in DNA.

In addition to the effect of ionizing radiation, we also examined the effect of H₂O₂ on formation of 8-OH-dG in DNA *in vivo*. H₂O₂ is known to have mutagenic (9), clastogenic (10) and carcinogenic (11) activities. It induced DNA damage *in vivo* such as strand scissions and thymine glycol formation (12). We previously observed the formation of 8-OH-dG on treatment of calf thymus DNA with H₂O₂ *in vitro* (3). In the present experiment, cells of *S. typhimurium* strain TA100 were treated with various concentrations of H₂O₂. As shown in Figure 5, the content of 8-OH-dG in their DNA increased with increase in the concentration of H₂O₂.

Discussion

The sensitive assay procedure recently developed by Floyd *et al.*, using an EC detector coupled with an h.p.l.c. apparatus (7) was found to be very useful for measurement of small amounts of 8-OH-dG in DNA. On treatment of cells or mice with oxygen radical producing agents, considerable portions of the cells may be killed or become non-functional. However, repair of 8-OH-dG in liver DNA after γ-ray irradiation was observed in mice, indicating that 8-OH-dG is formed in intact cells. The results shown in Figure 3 were obtained by irradiation for several hours at a low dose rate of 130 rad/min. Therefore, some repair may have occurred during irradiation, and the true yield of 8-OH-dG may have been higher, as indicated from the results at a high dose rate shown in Figure 4 (yield of 8-OH-dG: 0.032 residue/10⁵

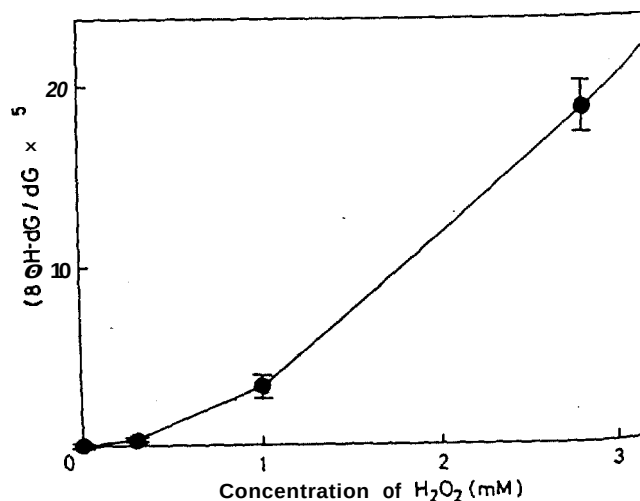


Fig. 5. Formation of 8-OH-dG in DNA of *S. typhimurium* TA100 cells treated with H₂O₂.

dG/krad). Repair of 8-OHdG may be related to unscheduled DNA synthesis, which was observed in mouse skin after ionizing irradiation (13).

A small amount of 8-OH-dG (0.6–1.4 residue/ 10^5 dG) was also detected in control DNA isolated from HeLa cells, mouse liver and *S. typhimurium* that had not been treated with oxygen radical-producing agents. These results suggested that in normal conditions cellular DNA is damaged with formation of 8-OH-dG residues by oxygen radicals that are generated by normal cellular metabolism. This is compatible with the fact that another product of DNA damage, thymine glycol, is excreted in the urine of normal rats and humans (14). Mutations were also observed in *Salmonella* strains TA2662 and TA103 recovered from the liver and spleen of mice, suggesting that oxygen radicals act as mutagens in these normal tissues (15).

It should be mentioned that on isolation of DNA with phenol that has not been freshly distilled the 8-OH-dG content of the DNA is substantially increased. In this case 8-OH-dG is presumably produced during isolation of DNA by oxidizing components present in the phenol. However, on DNA extraction with freshly distilled phenol, the 8-OH-dG content was the same as that obtained using Marmur's method employed in this work. Although we speculate that 8-OHdG is actually present in cellular DNA, the possibility cannot be completely excluded even with Marmur's method that it is an artifact produced in DNA. For example, peroxides are reported to be formed on incubation of homogenized mammalian tissues (16), and we observed that the 8-OH-dG content increased on prolonged homogenization of mouse liver.

Formation of 8-OH-dG in DNA is three orders of magnitude less on ionizing irradiation *in vivo* than on irradiation *in vitro* (2). Thus in intact cells there must be some mechanism for preventing formation of 8-OH-dG in DNA by oxygen radical-forming agents. The conformation of DNA in the nucleosome form may be more resistant to the agents, or some cellular components may inhibit the action of oxygen radicals. Another possibility is that there is an active repair mechanism for converting 8-OH-dG to dG in the cells. Further work is needed on this problem.

The extent of formation of 8-OH-dG in cellular DNA by ionizing radiation (0.008–0.032 residue/ 10^5 dG/krad) observed in this study seems to be of roughly the same order as that of thymine glycol formation (0.034 residue/ 10^5 dT/krad, calculated from data in ref. 17). Therefore, formation of 8-OH-dG in cellular DNA should be considered as a possible mechanism of involvement of oxygen radical-forming agents in mutation and carcinogenesis.

Acknowledgements

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