5-1) 8-HYDROXYGUANINE, A DNA ADDUCT FORMED BY OXYGEN RADICALS: ITS IMPLICATION ON OXYGEN RADICAL-INVOLVED MUTAGENESIS/CARCINOGENESIS

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SUMMARY

Oxygen radicals have been suggested to be involved in mutation/carcinogenesis. The C-8 position of guanine residues in DNA is hydroxylated to produce 8-hydroxyguanine(8-OH-Gua) in DNA *in vitro* by various oxygen radical producing agents. The formation of 8-OH-Gua was also observed in cellular DNA *in vivo* by radiation or oxygen radical forming carcinogens. The 8-OH-Gua residue in DNA is often misread in the position of 8-OH-Gua residue itself but also at neighboring residues next to 8-OH-Gua. When second guanine in codon 12 was specifically replaced with 8-OH-Gua and transferred to NIH3T3, the recipient cells were transformed to malignant cell type. *E. coli* was found to contain an endonuclease which specifically recognizes 8-OH-Gua residue and cleave DNA strand before and after the modified base.

The data obtained imply that 8-OH-Gua formed in DNA in vivo is recognized as an abnormal modified base which, if not repaired, play a role in the mediation of oxygen radical-involved mutation/carcinogenesis.

Key Words: 8-hydroxyguanine, oxygen radicals, endonuclease, mutagenesis/carcinogenesis

INTRODUCTION

It has been suggested that oxygen radicals are involved in both initiation and promotion of carcinogenesis(1,2). Oxygen radicals are produced by mutagens/carcinogens in the environment, but are also formed during various kinds of cellular metabolism. We previously observed that the C-8 position of guanine residues in DNA is hydroxylated to produce hydroxyguanine(8-OH-Gua) in DNA *in vitro* by various oxygen radical-producing agents(3). The formation of 8-OH-Gua was

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also observed in cellular DNA when mice or rats were irradiated by ionizing radiation or administrated oxygen radical-forming carcinogens(4-6).

An important question is what is the biological effect of 8-OH-Gua in DNA. To answer this question, oligodeoxynucleotides containing 8-OH-Gua in a specific position were chemically synthesized and this DNA was used as a template for DNA synthesis *in vitro* to observe the effect of 8-OH-Gua on DNA polymerase reaction (7), and also used as a substrate for detection of any activity in *E. coli* to recognize 8-OH-Gua and cleave the strand near the 8-OH-Gua residue. To further investigate the involvement of 8-OH-Gua in mutagenesis/carcinogenesis, c-Ha-ras gene of which the second position of guanine in codon 12 was replaced with 8-OH-Gua was tested for its transforming activity by transfecting this gene to NIH3T3 cells as recipient cells. It was observed that the 8-OH-Gua in DNA was misread, *E. coli* has an endonuclease specifically recognizing 8-OH-Gua in duplex DNA and the 8-OH-Gua-containing c-Ha-ras gene produced malignant foci in NIH3T3 cells.

MATERIALS and METHODS

8-Hydroxydeoxyguanosine(8-OH-dG) used as a standard for an assay for 8-OH-dG in DNA was prepared from deoxyguanosine(dG) with the Udenfriend system as described previously(3). The oligodeoxynucleotides containing 8-OH-Gua in a specific position was chemically synthesized as previously reported(7).

Assay for 8-OH-Gua in DNA

Tissue or leukocytes were frozen at -80°C until the DNA isolation. Defrosted tissues were gently homogenized in a Teflon homogenizer for the minimum time(10-20 sec) in the absence of air. To that end, all the apparatus and solutions were saturated with argon gas before homogenation. DNA was isolated by Marmur's method, except that cells were lyzed by 2% sodium dodecylsulfate at 37°C for 30 min. The resulting deoxynucleoside mixture was assayed by an electrochemical(EC) detector as described previously(8). Here it is of note that 8-OH-Gua was assayed by measuring the amount of 8-OH-dG.

Assay for nicking activity

For detection of 8-OH-Gua-recognizing endonuclease in *E. coli* extract, any product resulting from cleavage around the site of 8-OH-Gua was searched from the reaction mixture using polyacrylamide gel electrophoresis. The protein samples were

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incubated with double stranded(ds) 46mer DNA containing 8-OH-Gua(ds 46G-OH) labeled at 5'-end of the 8-OH-Gua-containing strand in 20 μl of 50 mM Tris-HCl, pH 7.5, 50 mM KCl and 2 mM EDTA at 37°C for 1 hr. After the incubation, each reaction mixture was mixed with 100 μl of phenol-chloroform(1:1, v/v) and 80 μl of H_2O. After centrifugation, the aqueous layer(50 μl) obtained was mixed with 200 μl of 0.3 M sodium acetate containing 1 mM EDTA and yeast tRNA(0.4 mg/ml) and precipitated by adding 750 ml of ethanol(precooled at -20°C) at -70°C. The precipitate obtained was dissolved in 0.3 M sodium acetate, precipitated again by ethanol and washed two times with 80% cold ethanol. The pellet was dried in vacuo, dissolved in 10 μl of loading buffer [80% formamide(v/v), 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol(w/v) and 0.1% bromophenol blue(w/v)] and denatured by heating at 90°C for 3 min. Two microliters(approximately 1/10 of the substrate amount) was applied to 20% denaturing polyacrylamide gel for electrophoresis to analyze the amount of DNA fragment cleaved at the position of 8-OH-Gua.

RESULTS

Formation of 8-OH-Gua in DNA *in vitro*

When a solution of dG or DNA(double stranded and single stranded) was shaken with various oxygen radical-forming agents, the formation of 8-OH-Gua was always observed. Those oxygen radical-forming agents include reducing agents(3), X-ray(9), asbestos plus hydrogen peroxide(10), and polyphenol with hydrogen peroxide and ferric ion(11). It is interesting to note the 8-OH-Gua is the only major product produced from purine deoxynucleosides by oxygen radical-producing chemicals, while X-ray produces both 8-OH-Gua and 8-hydroxyadenosine(The content of 8-OH-Gua in DNA is estimated by measuring the amount of 8-OH-dG).

Formation of 8-OH-Gua in DNA *in vivo*

The formation of 8-OH-Gua in DNA has also been observed *in vivo*. Namely, when the whole body of mice was irradiated with γ-ray, the amount of 8-OH-dG residue in liver DNA of the irradiated mice was increased proportionally to the dose of γ-ray(Fig. 1 and Fig. 2). It should be noted that the amount of 8-OH-dG in DNA after *in vivo* irradiation were three orders of magnitude lower than those after *in vitro* irradiation of

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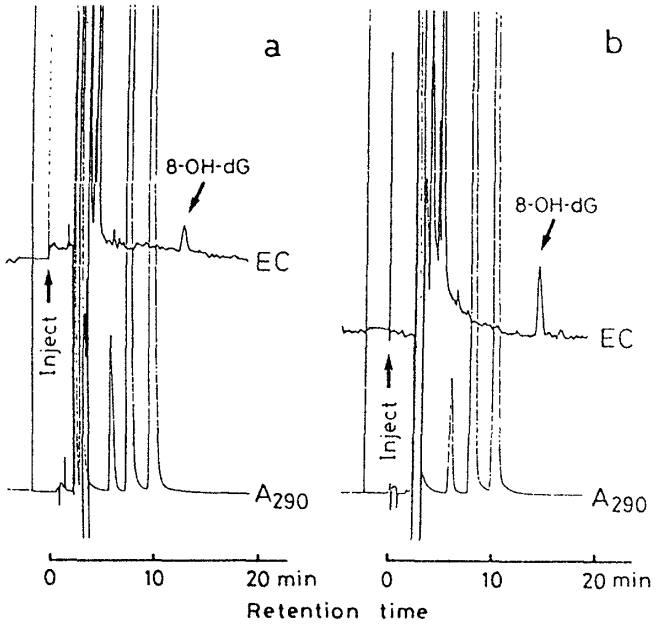


Fig. 1. Detection of 8-OH-dG in mouse liver DNA by HPLC coupled with an electrochemical detector. (a) Control mouse liver DNA; (b) liver DNA from a mouse irradiated with 173 Krad of γ -rays.

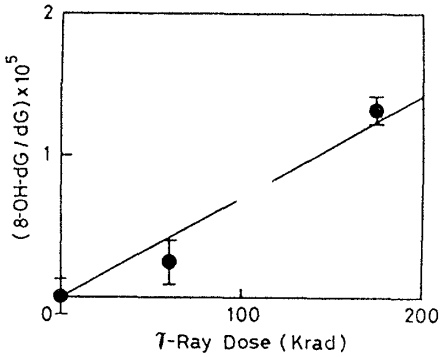


Fig. 2. Formation of 8-OH-dG in mouse liver DNA on whole body irradiation with γ -rays.

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DNA. Therefore the sensitive method to detect 8-OH-dG by using an electro-chemical detector was found to be very useful. It should be noted that extent of formation of 8-OH-Gua *in vivo* seems to be almost comparable to that of thymine glycol formation.

8-OH-Gua was also produced in DNA *in vivo* by administration of oxygen radical-producing carcinogens into rats. By oral administration of a renal carcinogen, potassium bromate($KBrO_3$), to the rat, a significant increase of 8-OH-Gua in kidney DNA was observed(5). Amount of 8-OH-Gua increased in kidney DNA is approximately 5 8-OH-Gua per 10^5 Gua molecule. On the contrary to kidney, the increase of 8-OH-Gua content in DNA of liver, a non-target tissue was not significant, suggesting that formation of 8-OH-Gua in tissue DNA is closely related to $KBrO_3$ carcinogenesis.

A similar result was obtained with another chemical carcinogen, ciprofibrate. Ciprofibrate is known to be a peroxisome proliferator that induces hepatocellular carcinoma in rats and mice. It was suggested that the proliferation of peroxisomes and induction of peroxisome-associated enzymes results in oxidative stress which then leads to tumorigenesis. J. K. Reddy and his colleagues, collaborating with us, have shown that administration of ciprofibrate in diet at a concentration of 0.025% for 16, 28, 36, or 40 weeks resulted in progressive increases in the level of 8-OH-Gua in rat liver DNA(6). The increase of 8-OH-Gua level seems to be attributed to persistant peroxisome proliferation resulting from chronic ciprofibrate treatment, since no increase in 8-OH-Gua was found in liver DNA of rats that received a single dose of ciprofibrate. Increased lipid peroxidation has been observed in liver of rats chronically treated with peroxisome proliferators(12). In this connection, it is interesting to note that 8-OH-Gua was formed when auto-oxidized linolenic acid or linoleic acid were reacted with dG or DNA(13).

Transforming activity of normal c-Ha-ras gene by replacing G in the second position of the 12th codon by 8-OH-Gua

An important question is whether the formation of 8-OH-Gua can be considered a likely cause of mutation or carcinogenesis by oxygen radicals. To investigate this possibility, we previously examined the effect of an 8-OH-Gua residue in DNA on the fidelity of DNA replication using a DNA synthesis system *in vitro* with *E. coli* DNA polymerase I(Klenow fragment)(7). The synthetic oligodeoxynucleotides, with or without an 8-OH-Gua residue in a specified position

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were used as templates for DNA synthesis under the conditions of the dideoxy chain termination sequencing method. It was shown that an 8-OH-Gua residue in DNA is misread to insert A, T, C and G with an almost equal frequency. In addition, pyrimidine residues next to the 8-OH-Gua were also misread(Fig. 3).

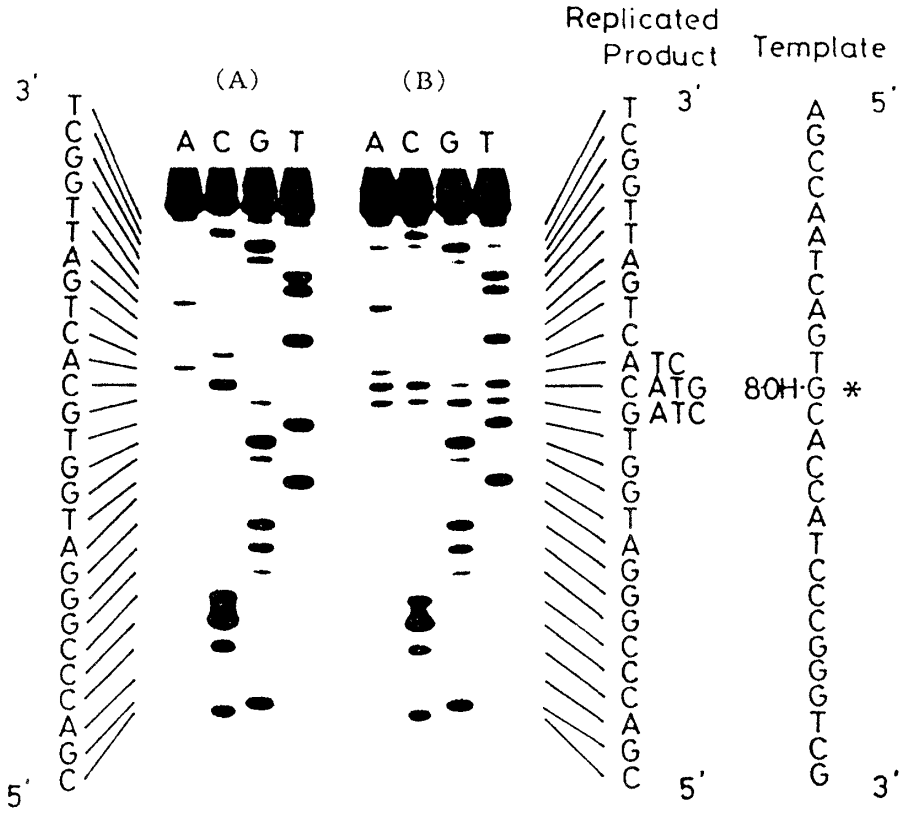


Fig. 3. Autoradiographs of 12% polyacrylamide sequencing gel, showing the sequence of the products synthesized under the direction of 8-OH-Gua containing DNA template, Fragment B. Fragment A is its counterpart containing normal Gua.

To further examine the effect of 8-OH-Gua in DNA in mutation, a synthetic normal c-Ha-ras gene having an 8-OH-Gua residue only at the second position of the 12th codon in place of G has been constructed and used for transfection of NIH3T3 cells. For this study, synthetic human c-Ha-ras genes(14) were ligated to the 3'-end or Rous sarcoma virus long terminal repeat(15). Also a vector containing the normal c-Ha-ras gene having GGT at the 12th codon was constructed. In addition, c-Ha-ras genes having either 8-

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OH-Gua or O^6-methylguanine(O^6-Me-Gua) at the second position of the 12th codon(GGT) was prepared by cassette mutagenesis.

Table 1. Transformation of NIH3T3 cells with synthetic c-Ha-ras gene containing 8-OH-Gua O^6-Me-Gua at codon 12

Amount of DNA/plate	Transformant/plate			
	Gly-12	Val-12	8-OH-Gua	O^6-Me-Gua
20 ng	0	101	0.2	2
50 ng	0	172	4.4	4

Gly-12, 5-GGT-3'
-CCA-;

Val-12 5'-GTT-3'
-CCA-;

8-OH-Gua or O^6-Me-dG, 5'-GGT*-3'
-CCA-

G* is modified base.

As shown in Table 1, a small number of transformed foci appeared with the 8-OH-Gua containing c-Ha-ras gene: the efficiency was approximately the same as that with the c-Ha-ras gene having O^6-Me-Gua. Although the further study is needed to identify the type of mutation present in the c-Ha-ras gene isolated from the transformed cells, this preliminary experiment suggests that 8-OH-Gua in DNA can induce mutation *in vivo*.

8-OH-Gua specific endonuclease from *E. coli*

The 8-OH-Gua produced in liver DNA by irradiation of mice decreased with time, suggesting the presence of a repair enzyme(s) acting on 8-OH-Gua in mouse liver(4). We have also noticed that the mean levels of 8-OH-Gua in peripheral blood leukocytes increased immediately after smoking, but rapidly returned to the original level(unpublished results). Thus an attempt has been made to isolate an enzyme from *E. coli* which specifically cleaves the site of an 8-OH-Gua residue in DNA. For this purpose, a double-stranded 46-mer having 8-OH-Gua was used as a substrate. We were able to detect an activity that cleaves the strand containing 8-OH-Gua, and have purified the enzyme without contamination of other DNases by successive

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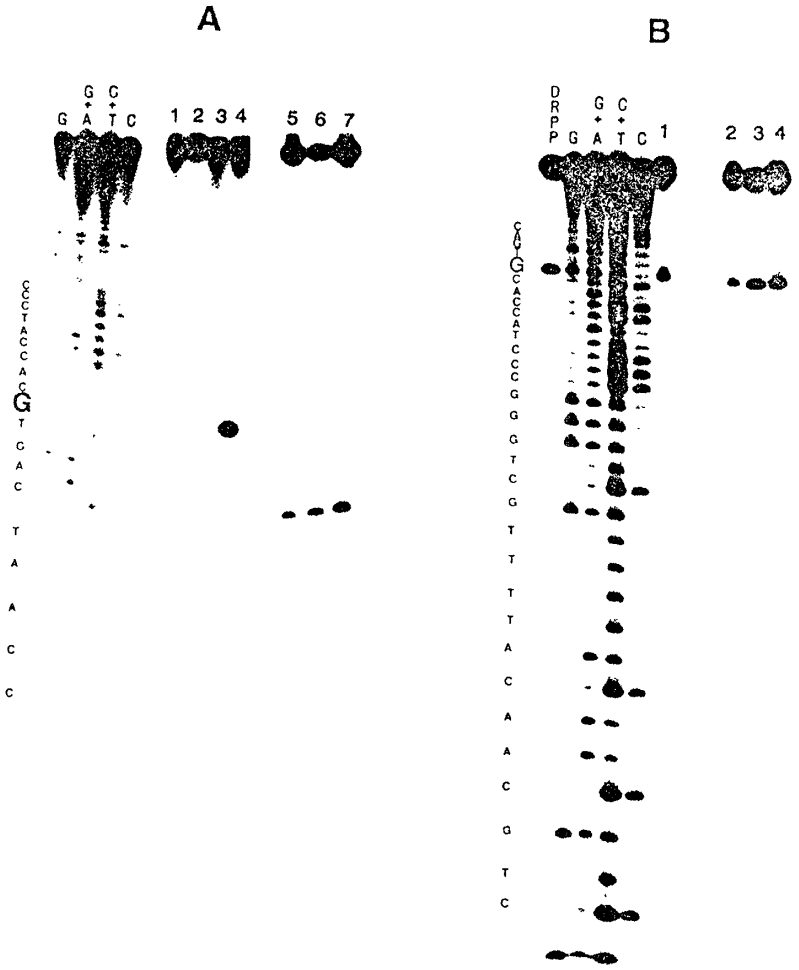


Fig. 4. Purified 8-OH-Gua endonucleases cleaving 8-OH-Gua-containing strand 5' and 3' to 8-OH-Gua. A. ds 46mer DNA(ds46) and ds46mer DNA containing 8-OH-Gua(ds46G-OH) labeled at the 5'terminus of the modified strand were incubated without(lanes 1 and 2, respectively) and with(lanes 3 and 4, respectively) 5 μl of enzyme solution. Lanes 5 and 6; aliquots of the lane 4 sample of this pannel and PPRD(hot piperidine-treated) ds46G-OH, lane 7; mixture of samples of Lanes 5 and 6. To the lanes 5,6 and 7, the longer electrophoresis was applied. The four lanes on the left are Maxam-Gilbert reactions of ss46 and bold-face G indicates the location of 8-OH-Gua. B. Experiments were performed as described in A except that ds46G-OH labeled at 3'-end of the modified strand was incubated with enzyme solution(lane 1). Lanes 2 and 3; aliquots of the lane 1 sample and the lane PPRD sample and lane 4; the mixture of lanes 2 and 3. The first lane on the left is 3'-labeled ss46G-OH treated with piperidine and the next four lanes, Maxam-Gilbert reactions of 3'-labeled ss46 mer. Bold-faced G indicates the position of 8-OH-Gua.

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purification procedures as ammonium sulfate precipitation, DEAE-cellulose and Sephadex G-100 column chromatography. The cleavage was observed only with double-standed DNA, but not with single-stranded DNA containing 8-OH-Gua. The enzyme cleaves both phosphodiester bonds, 3' and 5' to 8-OH-dG residue, leaving phosphate on the deoxyribose moiety of neighboring bases(Fig. 4). This endonuclease showed no activity on DNAs with mismatches, G:A and C:T. This enzyme was found to differ from *E. coli* endonuclease III and endonuclease IV. The enzyme may be a new endonuclease which specifically recognizes 8-OH-Gua in double-stranded DNA, thus implying that 8-OH-dG present in DNA is not artifically formed, but actually produced *in vivo* by oxygen radicals, and the cells acquire a mechanism to repair it.

DISCUSSION

Oxygen radicals induce to produce various kind of DNA damage such as DNA strand scission and formation of thymine glycol and 5-hydroxymethyluracil in addition to 8-OH-Gua formation(16). An important question is what type of DNA damage is relevant to mutagenesis/carcinogenesis caused by oxygen radicals. Much attention was paid previously to DNA strand scission and thymine glycol formation in this context, since those DNA damages were discovered many years ago, and extensively studied since then. However, as described in this article, it can be seen that 8-OH-Gua cannot be ignored when one considers the molecular mechanism of mutagenesis/carcinogenesis by oxygen radicals, although further studies are needed in order to get a clearer picture. In this connection, it is noteworthy to mention that point mutation in *E. coli*, *Neurospora crassa* and mammalian cells caused by γ-ray or X-ray irradiation was found to be GC → AT and GC → TA as well as AT → GC and AT → TA mutations, indicating that damage in a G:C pair is involved in mutagenesis(17-20).

The amount of 8-OH-Gua present in DNA is quite large as compared with formation of DNA adducts by alkylating agents or other chemical carcinogens(approximately $1/10^5$ against $1/10^8$ per Gua). One can argue that if 8-OH-Gua is really mutagenic, how cells can survive with such a high extent of modification. However, it is possible that the frequency of misreading of 8-OH-Gua found in *in vitro* DNA synthesis does not exactly represent the *in vivo* situation, since it is a model system using a single-stranded DNA as a template. The mutation frequency of 8-OH-Gua may be much smaller *in vivo*.

Quite a large amount of 8-OH-Gua is detected in DNA

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without treatment by oxygen radial forming agents. This background level of 8-OH-Gua cannot be diminished despite many precautions to avoid oxidation of DNA during isolation(approximately one 8-OH-Gua per 10^5 dG). A question is raised as to whether these 8-OH-Gua residues are endogeneously present in DNA, or produced artificially during the isolation process. Although a final answer has not yet been obtained, it should be mentioned that the amount of 8-OH-Gua present in human peripheral blood leukocytes DNA varied almost 10 folds depending upon the individual(1-10 8-OH-dG/10^5 dG). However it can also be argued that generation of H_2O_2 or other oxygen radical forming peroxides are produced metabolically *in vitro* after the cells are lysed, and such metabolic activities differ with each individual.

Perhaps the most important future approach to elucidate a mechanism of involvement of 8-OH-Gua in mutagensis/carcinogenesis is to further investigate mechanisms of repair of 8-OH-Gua in DNA in both bacteria and mammalian cells. Isolation of mutant(s) for repair of 8-OH-Gua should give more clear information on this interesting problem. In addition, studies on *in vivo* mutation by using a plasmid vector containing 8-OH-Gua in a specific position should be informative.

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