ACCELERATED PAPER

High accumulation of oxidative DNA damage, 8-hydroxyguanine, in Mmh/Ogg1 deficient mice by chronic oxidative stress

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8-Hydroxyguanine (8-OH-G) is a major pre-mutagenic lesion generated from reactive oxygen species. The Mmh/Ogg1 gene product plays a major role in maintaining genetic integrity by removing 8-OH-G by way of the base excision repair pathway. To investigate how oxidative stress influences the formation of 8-OH-G in Ogg1 mutant mice, a known oxidative agent, potassium bromate (KBrO3), was administered at a dose of 2 g/l in the drinking water to Ogg1+/+ and Ogg1-/— mice for 12 weeks. Apurinic (AP) site lyase activity, measured by the excision of 8-OH-G from synthetic oligonucleotides, remained unchanged in kidney cell extracts isolated from Ogg1 mutant mice when the mice were pre-treated by KBrO3. The levels of 8-OH-G in kidney DNA tremendously increased in a time-dependent manner following exposure of Ogg1+/— mice to KBrO3. Of particular note, the amount of 8-OH-G in kidney DNA from Ogg1+/— mice treated with KBrO3 was ~70 times that of KBrO3-treated Ogg1+/+ mice. The accumulated 8-OH-G did not decrease 4 weeks after discontinuing treatment with KBrO3. KBrO3 treatment for 12 weeks gave rise to increased mutation frequencies at the transgenic gpt gene in Ogg1+/+ mice kidney. Absence of the Ogg1 gene further enhanced the mutation frequency. Sequence data obtained from gpt mutants showed that the accumulated 8-OH-G caused mainly GC—TA transversion and deletion. Other mutations including GC—AT transition also showed a tendency to increase. These results indicate that 8-OH-G, produced by chronic exposure to exogenous oxidative stress agents, is not repaired to any significant extent within the overall genome of Ogg1+/— mice kidney.

Introduction

Oxidative DNA damage is caused by reactive oxygen species, which may be generated endogenously by cellular oxygen metabolism and exogenously by ionizing radiation, environmental mutagens and carcinogens. 8-Hydroxyguanine (8-OH-

Abbreviations: AP lyase, apurinic, apyrimidic lyase; Cm, chloramphenicol; 8-OH-G, 8-hydroxyguanine or 7,8-dihydro-8-oxoguanine; ECD, electrochemical detector; 6-TG, 6-thioguanine.
rates were higher in KBrO₃ treated Ogg₁⁻/⁻ mice compared with non-treated Ogg₁⁺/+ mice.

Materials and methods

Mice

The generation of Ogg₁ deficient mice by gene targeting in embryonic stem cells has been described previously (11). The Ogg₁⁺/+ mice (F₁ hybrid of 129sv and C57 BL/6J) were crossed with C57BL/6J mice or gpp transgenic mice of C57BL/6J background. The offspring were mated to obtain Ogg₁⁺/+, Ogg₁⁻/⁻, Ogg₁⁺/⁻, gpt/Ogg₁ and gpt/Ogg₁⁻/- mice. Mice were genotyped by PCR analysis of DNA isolated from tail tip. A combination of primer pairs were used to detect wild-type and mutant alleles: the primer pair 5'-CTC- ACTGAGGTCGCTGGACGAG-3' (M2) and 5'-CCATCTGTTGTCCTGCA-3' (M5) detect the wild-type allele generating a 318 bp fragment, whereas the primer pair 5'-AGTGCCGTGTCGACATGCAAGTA-3' (M11) and 5'-GTGTGCTCTGGATTTGACTCAGG-3' (M12) identify the mutant allele as a 394 bp product. The primers used to detect the presence of the gpt transgene were: 5'-GGCGAACCTATTATTCCTGCA-3' (gpt-l) and 5'-TGGAAACTATTTGACCCGCCTG-3' (gpt-2) which yield an amplification product of 590 bp.

Treatment of mice with KBrO₃

The levels of 8-OH-G and AP lyase activity were measured in Ogg₁⁺/+; Ogg₁⁻/⁻; and Ogg₁⁺/⁻ mice following KBrO₃ treatment. KBrO₃ solution (at a concentration of 2 g/l) was administered to mice (7–8 weeks old) in the drinking water for 1, 4, 8 and 12 weeks. Control mice were given distilled water and killed at the corresponding time points. A part of the Ogg₁⁺/+; Ogg₁⁻/⁻ and Ogg₁⁺/⁻ mice were treated with KBrO₃ for 4 weeks by followed distill water for 4 weeks. Three male and three female mice of each genotype were used at each of the time points. For gpt mutation assay, KBrO₃ solution at a concentration of 2 g/l was administered to gpt/Ogg₁⁺/+ and gpt/Ogg₁⁻/- mice in the drinking water for 12 weeks. Body weights were recorded three times per week and water consumption was measured weekly. Kidneys were isolated from the dead animals and stored at −80°C.

AP lyase assay

Ten to twenty milligram portions of kidney were homogenized in 10 µl/mg lysis buffer consisting of 50 mM HEPES (pH 7.0), 250 mM NaCl, 0.1% NP-40 and 1 mM phenylmethylsulfonyl fluoride and then centrifuged at 18 000 g for 30 min. The protein concentration of the supernatant fraction was measured using the BCA assay (Pierce, Illinois, US) and activity assays were performed using crude protein extracts. A 21-base oligonucleotide containing a single 8-OH-G (GOH), 5'-CAGCCAATCAGOHTGCACCATCC-3', was ³²P-labeled at the 5' terminus and annealed with a complementary oligonucleotide possessing a C base opposite the lesion. A total of 200 fmol of ³²P-labeled duplex oligonucleotide was incubated with 60 µg of crude protein extract in 25 µl of 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 5 mM EDTA and 1 µg poly(dA:dT) for 30 min at 37°C. After the reaction, the substrate and the cleaved products were precipitated with ethanol, separated by electrophoresis on a 20% polyacrylamide gel containing 7 M urea and detected using a bioimaging analyzer (BAS5000, Fuji Photo Film).

Measurement of 8-OH-G with HPLC-electrochemical detector (ECD)

Whole kidney was used as a tissue sample to measure the amount of 8-OH-G. Genomic DNA extraction, preparation of sample and measurement of 8-OH-G was performed as described previously (11).

gpt mutation assay

About one-half of a whole kidney was used to extract high molecular weight genomic DNA using the RecoverEase DNA Isolation Kit (Stratagene, California, US). Packaging of lambda phage DNA, infecting of the lambda phage to E.coli YG6020, plating of the E.coli and sequencing of the gpt gene from the resulting mutant colonies was performed according to the method of Nohmi et al. (15). To remove the possibility that mutational events could arise from clonal expansion, when the same mutations occurred more than one time in the same kidney it was counted as a single, independent mutation. The gpt mutation frequency was calculated by dividing the number of independent colonies resistant to chloramphenicol (Cm) and 6-thioguanine (6-TG) by the number of colonies resistant to Cm.

Results

AP lyase activity in kidney cells from Ogg₁ mutant mice

To investigate how oxidative stress influences the formation of 8-OH-G in Ogg₁ mutant mice, we selected KBrO₃ because it has been reported to induce large amounts of oxidative DNA damage when it was administered into rats (16,17). In addition, KBrO₃ is known to be a renal carcinogen of rats (18,19). KBrO₃ was administered at a dose of 2 g/l in the drinking water to Ogg₁⁺/+; Ogg₁⁻/⁻ and Ogg₁⁺/⁻ mice for 12 weeks. Three mice from each group were killed at each time point of 0, 1, 4, 8 and 12 weeks. A 200 fmol of 5'-³²P-labeled oligonucleotide containing a single 8-OH-G was annealed with its complementary oligonucleotide and incubated with 60 µg of crude protein from kidney extracts. The nicked product was measured by a bioimaging analyzer after 20% PAGE. Values are mean and SD.

Fig. 1. AP lyase activity in kidney cells. Water (open symbols) or 2 g/l KBrO₃ solution (filled symbols) was given to male Ogg₁⁺/+ (squares), Ogg₁⁻/⁻ (circles) and Ogg₁⁺/⁻ (triangles) mice for 12 weeks. Three mice from each group were killed at each time point of 0, 1, 4, 8 and 12 weeks. A 200 fmol of 5'-³²P-labeled oligonucleotide containing a single 8-OH-G was annealed with its complementary oligonucleotide and incubated with 60 µg of crude protein from kidney extracts. The nicked product was measured by a bioimaging analyzer after 20% PAGE. Values are mean and SD.
High accumulation of 8-OH-G in kidney DNA of Ogg1\(^{-/-}\) mice

To monitor the change of the 8-OH-G levels in Ogg1\(^{-/-}\) mutant mice under chronic oxidative stress condition, the amounts of 8-OH-G in three male and three female Ogg1\(^{-/-}\) mutant mice treated with KBrO\(_3\) for 1, 4, 8 and 12 weeks were measured with HPLC-ECD (Figure 2). The amount of 8-OH-G in kidney DNA from control Ogg1\(^{+/+}\) and Ogg1\(^{+-}\) mice was 2.2/10\(^6\) dG at each of the time points. The 8-OH-G levels in kidney DNA from mutant Ogg1\(^{-/-}\) mice increased gradually with time, reaching 13.6/10\(^6\) dG after 12 weeks. These results indicate that 8-OH-G accumulates spontaneously in kidney DNA of Ogg1\(^{-/-}\) mice as reported previously in the case of liver DNA of Ogg1\(^{-/-}\) mice (11).

The 8-OH-G levels in kidney DNA from Ogg1\(^{+/+}\) and Ogg1\(^{+-}\) mice were high over the 1–12 week period during treatment with KBrO\(_3\), giving rise to levels of 6.4 for Ogg1\(^{+/+}\) mice and 9.9 for Ogg1\(^{+-}\) mice/10\(^6\) dG. This difference suggests that 50\% of OGG1 enzyme activity is not sufficient to repair 8-OH-G efficiently under high oxidative stress conditions. The amounts of 8-OH-G in kidney DNA from Ogg1\(^{-/-}\) mice treated with KBrO\(_3\) increased in proportion to administration time and to our surprise reached levels of 457.3/10\(^6\) dG after 12 weeks. This amount is ~200 times that of non-treated Ogg1\(^{-/-}\) mice. This level corresponds to 1.5\times10^6 8-OH-G lesions in the diploid genome of kidney cells or one 8-OH-G every 4.4 kb of genomic DNA.

These results show that the accumulation of 8-OH-G in DNA of Ogg1\(^{-/-}\) mice is dependent upon the time that the mice have been exposed to oxidative stress.

Change of the 8-OH-G levels in kidney DNA after discontinuing KBrO\(_3\) treatment

To investigate whether the high levels of accumulated 8-OH-G would decrease after the cessation of KBrO\(_3\) treatment, we measured the amounts of 8-OH-G in kidney DNA from Ogg1\(^{-/-}\) mutant mice that were given water for 4 weeks following treatment with KBrO\(_3\) solution for 8 weeks (Figure 3). After discontinuing KBrO\(_3\) treatment, the amount of 8-OH-G in kidney DNA from Ogg1\(^{+/+}\) and Ogg1\(^{+-}\) mice decreased to levels similar to those seen in non-treated Ogg1\(^{+/+}\) and Ogg1\(^{+-}\) mice. Surprisingly, the highly accumulated levels of 8-OH-G in Ogg1\(^{+/+}\) mice kidney did not decrease during 4 weeks under normal conditions. These results indicate that OGG1 is the major enzyme in the repair of 8-OH-G within the overall genome, at least in kidney, which consists of slow proliferating cells.

Mutation frequency in kidney DNA

To study the effect of KBrO\(_3\) treatment on mutation induction in kidney DNA from Ogg1\(^{-/-}\) mutant mice, the mutation frequency in the inactive prokaryotic gpt gene was measured in gpt/Ogg1\(^{+/+}\) and gpt/Ogg1\(^{+-}\) mice treated with KBrO\(_3\) for 12 weeks. The number of gpt mutant colonies (6-TG-resistant and Cm-resistant) and the total number of colonies plated (Cm-resistant) and the mutation frequencies in each mice are shown in Table I. Sequence analysis of gpt mutant colonies was carried out to assess the types of mutations (Table II). Because identical mutations found within one animal were considered to result from clonal expansion, the mutation frequency was
calculated from independent mutations that were determined in mutants isolated from different animals.

The background mutation frequency measured in kidney DNA from Ogg1+/+ mice was in agreement with previous results from liver DNA (11). There was no significant difference between non-treated Ogg1+/+ and Ogg1−/− mice. When Ogg1+/+ mice were treated with KBrO₃, the mutation frequency showed a 2.2-fold increase (Fisher test, P < 0.05). Deficiency of the Ogg1 gene also affected the mutation frequency of the gpt gene in KBrO₃ treated groups. The mutation frequency in Ogg1−/− mice was 2.9 times higher than that of Ogg1+/+ mice. This increase was statistically significant (Fisher test, P < 0.05). These results indicate that the high accumulation of 8-OH-G in DNA causes increases in the mutation frequency.

**Mutation spectrum in kidney DNA**

As shown in Table II, the majority of mutations in the gpt gene recovered from non-treated mice were base substitutions. GC→TA transversion in Ogg1−/− mice (50%) was more frequent than that in Ogg1+/+ mice (5.9%). It seems that 8-OH-G accumulated spontaneously leads to an increase of GC→TA mutations in kidney. This mutation spectrum was similar to previous results from liver (11) although there was no difference of mutation frequency in kidney DNA between non-treated Ogg1+/+ and Ogg1−/− mice. In KBrO₃ treated mice treated with KBrO₃ for 12 weeks.
treated groups, GC→TA transversions and deletions occurred frequently. The frequency of these mutations in Ogg1+/− mice treated with KBrO₃ (11.1×10⁻⁶, 9.9×10⁻⁶) was especially high as compared with Ogg1+/+ mice (2.4×10⁻⁶, 2.8×10⁻⁶). This indicates that GC→TA transversions and deletions were caused by 8-OH-G that accumulated to high levels in kidney DNA of Ogg1+/− mice. Further, we found a tendency for the increase of other mutation frequencies in treated Ogg1+/− mice, especially GC→AT. It seems that a high amount of 8-OH-G in the genome not only causes some base substitution mutations but also enhances deletion mutations.

Discussion

In the present paper, we demonstrated that the 8-OH-G level in kidney DNA from Ogg1 deficient mice increased as a result of chronic oxidative stress and that the accumulated 8-OH-G failed to be repaired even after the mice were returned to non-stress conditions for 4 weeks. We have also analyzed the amount of 8-OH-G in liver of Ogg1+/− mice after chronic exposure to KBrO₃ for 12 weeks. The amount of 8-OH-G in liver DNA increased to 96.6/10⁶ dG, >25-fold as compared with that of Ogg1+/+ mice treated with KBrO₃. The amount of 8-OH-G in liver DNA did not decrease after termination of exposure to KBrO₃ as in the case of kidney DNA (unpublished data). Thus it is likely that OGG1 plays a major role in repair of 8-OH-G not only in kidney but also other organs. Reactive oxygen species are generated endogenously by cellular metabolism or exogenously by environmental mutagens and carcinogens. Our experiments showed that the accumulation of 8-OH-G depends upon the exposure dosage to oxidative stress from exogenous sources in Ogg1 deficient mice. Furthermore, the exogenous oxidative stress causes an increase in the mutation frequency of Ogg1 deficient mice.

It was reported previously that an Ogg1+/− mouse embryonic fibroblast cell line can repair 8-OH-G that has been produced after cells have been exposed to photosensitizer in the presence of light (12,14). The repair rates were similar between dividing and non-dividing cells. This might suggest that other back-up systems exist to repair 8-OH-G. Indeed, Hazra et al. have found an OGG-2 enzyme, which cleaves 8-OH-G/G and 8-OH-G/A lesions more efficiently than 8-OH-G/C (20). Recently the same group identified another repair enzyme, NEH1, which was shown to have a wide substrate specificity (21). Interestingly, the nucleotide excision repair pathway may also play a role in the repair of 8-OH-G because the 8-OH-G base was removed from synthetic oligonucleotides by a reconstituted system consisting of XPA, RPA, TFIIH, XPC-HHR23B, XPG and XPF-ERCC1 (22). In our study the amount of 8-OH-G did not decrease in kidney DNA from Ogg1+/− mice 4 weeks after discontinuing treatment with KBrO₃. Therefore, it is clear that the contribution of other enzymes to the removal of 8-OH-G within the overall genome is marginal, at least in the slow proliferating kidney cells. Thus, the Ogg1 deficient mouse is a good animal model to monitor the levels and distribution of oxidative damage in various mouse tissues by measuring 8-OH-G levels after exposure to an exogenous genotoxic agent.

Recently Le Page et al. reported that when positioned on the transcribed strand 8-OH-G inhibits transcription by RNA polymerase II. Furthermore, they showed that 8-OH-G is removed more efficiently from the transcribed strand than the non-transcribed strand by the process of transcription-coupled repair (TCR) (23). TCR requires factors such as TFIIH, XPG, CSB, MSH2 and BRCA1 (24–26). TCR can remove 8-OH-G in the transcribed strand of Ogg1+/− cell lines, although slower than that of Ogg1+/+ cell lines (27). This may explain why the high accumulation of 8-OH-G observed in this study did not lead to disease or death over the time period analyzed. The above report provided the evidence that 8-OH-G positioned on the non-transcribed strand of a shuttle vector is not repaired in Ogg1+/− cell lines (27). This observation taken together with our result suggested that 8-OH-G produced by KBrO₃ in Ogg1+/− mice might accumulate exclusively in the non-transcribed strand.

In our study, the KBrO₃ treatment of Ogg1+/+ and Ogg1+/− mice did not alter the AP lyase activities in kidney cells. In contrast, Lee et al. showed that 8-OH-G glycosylase activity in kidney was induced after intraperitoneal KBrO₃ treatment of rats (28). It is possible that although OGG1 enzyme activity might be induced temporarily by oxidative stress, induction of the activity may not persist in the longer term under chronic oxidative stress.

The high accumulation of 8-OH-G, mispairing potentially with A, in kidney DNA of Ogg1+/− mice treated with KBrO₃ does not appear to be compatible with a low mutation rate. The fixation of mutation is limited because only a small fraction of kidney cells could proliferate over the 12 week period. Great increases in the mutation rate may result from an extension of the duration of the experiment or an induction of cell proliferation. It seems possible that there are additional mechanisms to the OGG1 enzyme that can protect the genome from the oxidative DNA damage, 8-OH-G. In this respect, the homolog of the MutY DNA glycosylase might play a key role (6). This enzyme excises A when mispaired with 8-OH-G and thereby allows a C base to be incorporated correctly. It may also explain the low mutation rates observed since the translesion synthesis by DNA polymerase η appeared to replicate DNA accurately by inserting C opposite 8-OH-G (29). Furthermore, replication coupled repair of 8-OH-G might have contributed to the observed results (21).

It remains to be solved whether a high amount of 8-OH-G in genomic DNA of proliferating or non-proliferating tissue cell might cause carcinogenesis, aging and other diseases. In this connection it should be noted that no renal tumor was formed thus far in Ogg1+/− mice after 12 weeks exposure to KBrO₃ (unpublished data). To clarify the link between mutation and carcinogenesis with 8-OH-G, we are planning to perform carcinogenesis tests with Ogg1 mutant mice in a more detailed manner and to analyze various double knockout mice strains.

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References


