[CANCER RESEARCH 63, 4287–4292, July 15, 2003]

Cell Proliferation in Liver of *Mmh/Ogg1*-deficient Mice Enhances Mutation Frequency because of the Presence of 8-Hydroxyguanine in DNA

Tsuyoshi Arai, Vincent P. Kelly, Kimiyo Komoro, Osamu Minowa, Tetsuo Noda, and Susumu Nishimura[1]

Banyu Tsukuba Research Institute in Collaboration with Merck Research Laboratories, Tsukuba, Ibaraki 300-2611 [T. A., V. P. K., K. K., S. N.]; Department of Cell Biology, The Cancer Institute, Japanese Foundation for Cancer Research, Toshima-Ku, Tokyo 170-8455 [T. A., O. M., T. N.]; Mouse Functional Genomics Research Group, RIKEN Genomic Sciences Center, Totsuka-ku, Yokohama, Kanagawa 244-0804 [O. M.]; CREST, Japan Science and Technology Corporation, Kawaguchi 332-0012 [O. M., T. N.]; and Department of Molecular Genetics, Tohoku University School of Medicine, Aoba-Ku, Sendai 980-8575 [T. N.], Japan

ABSTRACT

The *Mmh/Ogg1* gene product maintains the integrity of the genome by removing the damaged base 8-hydroxyguanine (8-OH-G), one of the major DNA lesions generated by reactive oxygen species. Using *Ogg1*-deficient mice, we sought to establish if cells having high amounts of 8-OH-G have the ability to proliferate and whether the mutation frequency increases after proliferation *in vivo*. When $KBrO_3$, a known renal carcinogen, at a dose of 2 grams/liter was administered to *Ogg1* mutant mice for 12 weeks, the amount of 8-OH-G in liver DNA from treated $Ogg1^{-/-}$ mice increased 26.1 times that of treated $Ogg1^{+/+}$ mice. The accumulated 8-OH-G did not decrease 4 weeks after cessation of $KBrO_3$ treatment. Partial hepatectomy was performed on $Ogg1^{+/-}$ and $Ogg1^{-/-}$ mice after being treated with $KBrO_3$ for 12 weeks. The remnant liver from $Ogg1^{-/-}$ mice treated with $KBrO_3$ regenerated to the same extent as nontreated $Ogg1^{+/-}$ mice. In addition, 8-OH-G was not repaired during cell proliferation by partial hepatectomy, indicating that there is no replication coupled repair of preexisting 8-OH-G. The mutation frequency after the regeneration of liver from treated $Ogg1^{-/-}$ mice showed a 3.5-fold increase compared with before regeneration. This represents a mutation frequency 6.2 times that of normal levels. The proliferation of cells having accumulated amounts of 8-OH-G caused mainly GC→TA transversions. These results showed that inactivation of the *Ogg1* gene leads to a higher risk of cancer because cells with accumulated 8-OH-G still retain the ability to proliferate, leading to an increase in the mutation frequency.

INTRODUCTION

Reactive oxygen species, which are generated endogenously by cellular oxygen metabolism or exogenously by ionizing radiation, environmental mutagens, and carcinogens produce many types of DNA damage, including base and sugar modifications, DNA–protein cross-linking, and strand breaks. 8-OH-G[2] is a major form of oxidative DNA damage and believed to contribute to mutagenesis, carcinogenesis, and aging (1). To date, the majority of studies has focused on the effect of 8-OH-G *in vitro* or with tissue culture cells, which have shown, by using various DNA polymerases or shuttle vectors, that 8-OH-G primarily causes GC→TA transversions as a result of mispairing with an A base (2–10).

In the mammalian cell, the *MMH/OGG1* gene encodes for a DNA glycosylase/AP lyase that has the capacity to excise 8-OH-G from DNA (11–17). The OGG1 protein initiates the base excision repair pathway by recognizing and excising the oxidative DNA lesion. In addition to OGG1, the mammalian cell contains two further enzymes, MYH and MTH, that contribute to the protection from 8-OH-G damage. MYH is a monofunctional glycosylase that removes an A base that has mispaired with 8-OH-G (18). MTH is an 8-OH-dGTPase that degrades 8-OH-dGTP in the nucleotide pool, thereby preventing its incorporation into DNA (19). The importance of the 8-OH-G repair systems is reflected in the fact that homologues have been identified in various species from prokaryotes to eukaryotes.

With respect to involvement of the *OGG1* gene in human cancer, significant effort has been made to link the inactivation of the *OGG1* gene with cancer risk in clinical tumor samples (20–24). However, the data, such as somatic mutation, loss of heterozygosity, and polymorphism, are contradictory. Moreover, it remains to be firmly established that the accumulation of 8-OH-G causes carcinogenesis by using experimental animal models. In this study, we showed that 8-OH-G accumulated in liver DNA of $Ogg1^{-/-}$ mouse treated with $KBrO_3$, which is a known carcinogen and oxidative agent for rat kidney (25–28), and persisted in the same level after cessation of $KBrO_3$ treatment as in the case of kidney DNA as reported previously (29). Furthermore, after partial hepatectomy, liver with such oxidative DNA lesions regenerated normally without repair of 8-OH-G, followed by a considerable increase in the mutation frequency. These results indicated that there is no major replication coupled repair of preexisting 8-OH-G. Because induction of carcinogenesis must be coupled with cell proliferation, this system would be a good animal model for long-term carcinogenesis study to elucidate the involvement of 8-OH-G.

MATERIALS AND METHODS

Mice. The generation of *Ogg1*-deficient mice has been described elsewhere (30). The $Ogg1^{+/-}$ mice, which were F1 hybrid of 129sv and C57BL/6J, were crossed with C57BL/6J or *gpt* transgenic mice of C57BL/6J background. The offspring were mated and $Ogg1^{+/+}$, $Ogg1^{+/-}$, $Ogg1^{-/-}$, *gpt*/$Ogg1^{+/+}$, *gpt*/$Ogg1^{+/-}$, and *gpt*/$Ogg1^{-/-}$ mice were obtained. In the $KBrO_3$ treatment time course experiments, $Ogg1^{+/+}$, $Ogg1^{+/-}$, and $Ogg1^{-/-}$ mice were used. For partial hepatectomy experiments, *gpt*/$Ogg1^{+/-}$ and *gpt*/$Ogg1^{-/-}$ mice were obtained by crossing *gpt*/$Ogg1^{+/-}$ and $Ogg1^{-/-}$ mice. Genotyping of the mice was performed by PCR analysis of DNA isolated from tail tips (29).

Time Course Experiment of $KBrO_3$ Treatment. Seven to 8-week-old $Ogg1^{+/+}$, $Ogg1^{+/-}$, and $Ogg1^{-/-}$ mice were prepared. $KBrO_3$ was administered at a dose of 2 grams/liter in the drinking water to *Ogg1* mutant mice. Three male and three female *Ogg1* mutant mice of each genotype were killed at each time point of 1, 4, 8, and 12 weeks. Control mice were given water and killed at the same time point. A proportion of the *Ogg1* mutant mice was given water 4 weeks after cessation of $KBrO_3$ treatment for 8 weeks and killed. The livers were excised from the sacrificed mice and stored at −80°C before measurement of the 8-OH-G levels.

Partial Hepatectomy Experiment of Mice Treated with $KBrO_3$. In this experiment, male *gpt*/$Ogg1^{+/-}$ and *gpt*/$Ogg1^{-/-}$ mice at the age of 7–8 weeks old were used. $KBrO_3$ was administered at a dose of 2 grams/liter to three mice of each genotype for 12 weeks. The mice were kept for 1 week after treatment with $KBrO_3$ before partial hepatectomy. Partial hepatectomy, involving the removal of the anterior and left lateral hepatic lobes, was performed by the procedure of Higgins and Anderson (31). At the time of partial hepatectomy, the resected liver was weighed, and this information was used to calculate the remnant liver weight for each mouse based on the assumption that the remnant liver = (the resected liver) × 3/7. All mice were killed 10 days later. On sacrifice, the remnant liver was weighed, and the proliferation ratio of the

Received 2/16/03; accepted 4/30/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Banyu Tsukuba Research Institute in Collaboration with Merck Research Laboratories, 3 Okubo, Tsukuba, Ibaraki 300-2611 Japan. Phone: 81-298-77-2000; Fax: 81-298-77-2034; E-mail: nismrasm@banyu.co.jp.

[2] The abbreviations used are: 8-OH-G, 8-hydroxyguanine; HPLC, high-performance liquid chromatography; 6-TG, 6-thioguanine; Cm, chloramphenicol.

remnant liver was calculated. The isolated liver was stored at −80°C before analysis of the 8-OH-G levels and mutation.

Measurement of the 8-OH-G Level. About 100–200 mg of liver were used for the measurement of 8-OH-G levels using an HPLC-electrochemical detector. Genomic DNA extraction, preparation of the sample, and measurement of 8-OH-G were performed as described previously (30).

gpt Mutation Assay. High molecular weight genomic DNA was extracted from 50–80 mg of liver using the RecoverEase DNA Isolation Kit (Stratagene). The *gpt* mutation assay was performed according to the procedure of Nohmi *et al.* (32). Briefly, extracted genomic DNA was packaged into λ phage. The phage was infected to *Escherichia coli* YG6020 and plated, and the sequence of the *gpt* gene from the resulting mutant colonies was obtained. To remove the possibility that mutational events might arise from clonal expansion, identical mutations occurring more than once in the same liver sample were counted as a single, independent mutation. The *gpt* mutation frequency was calculated by dividing the number of independent colonies resistant to Cm and 6-TG by the number of colonies resistant to Cm alone.

Statistics. The significance of differences in the measured parameters was evaluated by using the Student *t* test or ANOVA followed by Scheffe multiple comparison test.

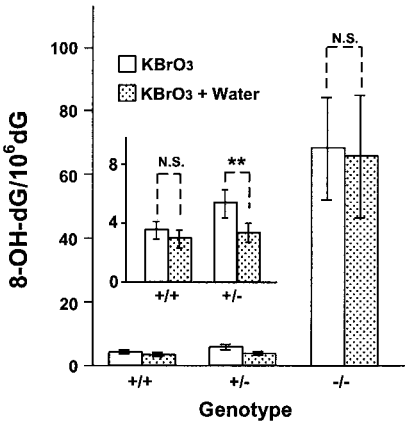

Fig. 2. The 8-OH-G levels in liver DNA after discontinuing $KBrO_3$ treatment. After administration of 2 grams/liter $KBrO_3$ solution to $Ogg1^{+/+}$, $Ogg1^{+/-}$, and $Ogg1^{-/-}$ mice for 8 weeks (*open bars*), water was given for an additional 4 weeks (*dotted bars*). The amount of 8-OH-G in liver DNA from three male and three female mice in each group was measured with HPLC-electrochemical detector. Values are mean and SD. *Asterisks,* significant difference at $P < 0.01$. *N.S.,* no significant difference.

RESULTS

The Ability of $KBrO_3$ to Produce 8-OH-G in Liver DNA. $KBrO_3$ is a known renal mutagen and carcinogen. It was unknown whether $KBrO_3$ can cause oxidative damage to DNA in other tissues, *e.g.,* liver. The analysis of oxidative damage to liver DNA is important because liver is susceptible to oxidative stresses such as that are produced during xenobiotic metabolism. We measured the amount of 8-OH-G in liver DNA from $Ogg1^{+/+}$, $Ogg1^{+/-}$, and $Ogg1^{-/-}$ mice to which $KBrO_3$ was administered at a dose of 2 grams/liter in the drinking water for 12 weeks. Three male and three female mice were analyzed at each time point. There was no observable histological difference in the livers of all groups in sections stained with H&E (data not shown).

The 8-OH-G levels of liver DNA from male and female mice within the same group were similar, shown together in Fig. 1. The amount of 8-OH-G in liver DNA from both control $Ogg1^{+/+}$ and $Ogg1^{+/-}$ mice was constant at $2.6/10^6$ dG after 12 weeks. In control $Ogg1^{-/-}$ mice, the amount increased proportionally with time, from 8 to $15.3/10^6$ dG. At 12 weeks, equal to 19–20 weeks old, the 8-OH-G level of control $Ogg1^{-/-}$ mice was 5.9 times that of control. These results are in good agreement with those of our previous study (30). When *Ogg1* mutant mice were treated with $KBrO_3$, the amount of 8-OH-G in liver DNA from $Ogg1^{+/+}$ and $Ogg1^{+/-}$ mice increased during the first 4 weeks but thereafter remained mainly unchanged from 4 to 12 weeks. The averages were 3.7 and $5/10^6$ dG, respectively. The absence of OGG1 enzyme caused a high accumulation of 8-OH-G in liver DNA from $Ogg1^{-/-}$ mice treated with $KBrO_3$. The amount of 8-OH-G tremendously increased in proportion to time with $KBrO_3$ treatment and reached $96.6/10^6$ dG. This represents a 37.2-fold increase over control $Ogg1^{+/+}$ mice and 26.1-fold increase compared with $KBrO_3$-treated $Ogg1^{+/+}$ mice. These results show that, in mice, $KBrO_3$ produces 8-OH-G in liver DNA, in addition to kidney DNA (29), and that the 8-OH-G in liver DNA of $Ogg1^{-/-}$ mice accumulates because of chronic oxidative stress.

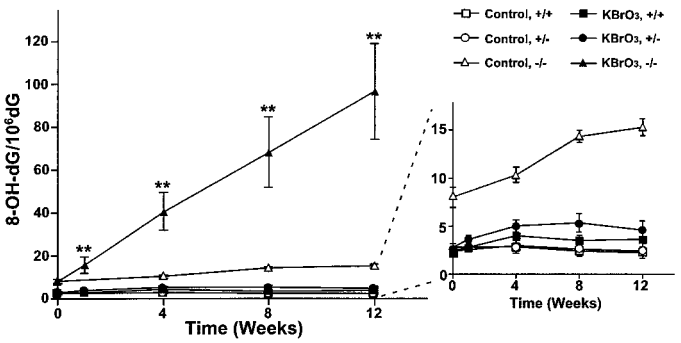

Fig. 1. The ability of $KBrO_3$ to produce 8-OH-G in liver DNA *in vivo*. Water (*open symbols*) or 2 grams/liter $KBrO_3$ solution (*filled symbols*) were given to $Ogg1^{+/+}$ (*squares*), $Ogg1^{+/-}$ (*circles*), and $Ogg1^{-/-}$ (*triangles*) mice for 12 weeks. The amount of 8-OH-G in liver DNA from three male and three female mice in each group was measured with HPLC-electrochemical detector. *Left panel,* all results. *Right panel,* a magnified part of the *left panel,* except for $Ogg1^{-/-}$ mice treated with $KBrO_3$. Values are mean and SD. The amount of 8-OH-G in liver DNA from $Ogg1^{-/-}$ mice treated with $KBrO_3$ was significantly higher than those of control $Ogg1^{-/-}$ mice at $P < 0.01$.

8-OH-G Levels in Liver DNA after Discontinuing $KBrO_3$ Treatment. We investigated how the levels of 8-OH-G in liver DNA from *Ogg1* mutant mice may be affected after cessation of $KBrO_3$ treatment. $Ogg1^{+/+}$, $Ogg1^{+/-}$, and $Ogg1^{-/-}$ mice were given 2 grams/liter $KBrO_3$ solution in the drinking water for 8 weeks; the treatment was discontinuing for 4 weeks before sacrifice and analysis of the mice (Fig. 2). Three male and three female mice were killed at each point. In $Ogg1^{+/+}$ and $Ogg1^{+/-}$ mice liver DNA, the amount of 8-OH-G decreased to control levels (2.9 and $3.3/10^6$ dG, respectively). By contrast, the accumulated 8-OH-G ($68.3/10^6$ dG) in liver DNA from $Ogg1^{-/-}$ mice did not change ($65.6/10^6$ dG) after the cessation of $KBrO_3$ treatment. It appears that the 8-OH-G accumulated in liver by chronic oxidative stress cannot be repaired in $Ogg1^{-/-}$ mice. These results show that the major enzyme to repair 8-OH-G in liver is the *Ogg1* gene product, which is in good agreement with our previous observations in kidney (29).

Regeneration of *Ogg1* Mutant Mice Liver after Partial Hepatectomy. The ability of cells to proliferate, after accumulating high amounts of 8-OH-G, was investigated by performing partial hepatectomy after $KBrO_3$ treatment. In addition, the levels of 8-OH-G and mutation frequency were taken after cell proliferation. $Ogg1^{+/-}$ mice were used as control instead of $Ogg1^{+/+}$ mice because only a slight difference exists in the 8-OH-G levels after $KBrO_3$ treatment, and in addition, similar to $Ogg1^{+/+}$ mice, the 8-OH-G level in $Ogg1^{+/-}$ mice liver decreased to control level after cessation of treatment with $KBrO_3$ (Figs. 1 and 2). Only male mice were used to remove the effect of sex differences on cell proliferation after partial hepatectomy. $KBrO_3$ was administered at a dose of 2 grams/liter in the drinking water to $Ogg1^{+/-}$ and $Ogg1^{-/-}$ mice for 12 weeks. Partial hepatectomy was performed 1 week after discontinuing treatment with $KBrO_3$. All mice were killed 10 days after the partial hepatectomy.

We did not find any histological difference in regenerated liver sections stained with H&E from *Ogg1*$^{+/-}$ and *Ogg1*$^{-/-}$ mice 10 days after partial hepatectomy (data not shown). The weight of the remnant liver from all mice increased 2.2–2.4 times after 10 days (Fig. 3). These results indicate that liver with high amounts of 8-OH-G can proliferate normally.

The Effect of Cell Proliferation on the Levels of 8-OH-G in Liver DNA. In control and $KBrO_3$-treated *Ogg1*$^{+/-}$ mice, the amounts of 8-OH-G in liver resected at partial hepatectomy were the same (2.4 and 2.2/10^6 dG, respectively; Fig. 4). After liver regeneration, there were no differences in the 8-OH-G levels in the mice as compared with before liver regeneration. In control *Ogg1*$^{-/-}$ mice, the 8-OH-G level, at 16.4/10^6 dG, decreased to 10.6/10^6 dG after liver regeneration ($P < 0.05$). Furthermore, in *Ogg1*$^{-/-}$ mice treated with $KBrO_3$, the 8-OH-G level, at 113.5/10^6 dG, decreased to 47.1/10^6 dG ($P < 0.01$). The decreases in 8-OH-G levels observed after liver regeneration were 64.6 and 41.5% in control and $KBrO_3$-treated *Ogg1*$^{-/-}$ mice, respectively. It appears that the decreases are related to the amount of liver proliferation. These results show that, after cell proliferation, the accumulated 8-OH-G levels in liver are diluted by a factor of two, indicating that 8-OH-G is not removed during cell proliferation.

Mutation Frequency in Liver DNA after Partial Hepatectomy. To investigate whether high amounts of 8-OH-G in tissue DNA can cause mutation *in vivo,* we measured the mutation frequency of the transgenic *gpt* gene in *gpt*/*Ogg1*$^{+/-}$ and *gpt*/*Ogg1*$^{-/-}$ mouse liver (Table 1). In control *Ogg1*$^{+/-}$ mice, the mutation frequency showed no difference before or after liver regeneration. The mutation frequency of control *Ogg1*$^{-/-}$ mice seemed higher than that of control *Ogg1*$^{+/-}$ mice, in agreement with our previous result (30), and showed a tendency to increase after liver regeneration. When *Ogg1*$^{+/-}$ and *Ogg1*$^{-/-}$ mice were treated with $KBrO_3$, we found no large increase in the mutation frequencies compared with each control. It is likely that $KBrO_3$ produces no mutation in mouse liver during 12 weeks. In liver DNA from *Ogg1*$^{-/-}$ mice treated with $KBrO_3$, the mutation frequency (10.1×10^{-6}) substantially increased, 3.5 times (35.4×10^{-6}), after liver regeneration over 10 days ($P < 0.03$). This mutation frequency was 6.2-fold that of control *Ogg1*$^{+/-}$ mice ($P < 0.01$). The results indicate that cell proliferation plays a pivotal role in fixing the mutation in tissue with a high accumulation of 8-OH-G.

Mutation Spectrum in Liver DNA after Partial Hepatectomy. The mutation spectrum in the *gpt* gene of mutant colonies is shown in Table 2. We found no difference in the mutation frequency and spectrum before and after liver regeneration in *Ogg1*$^{+/-}$ mice.

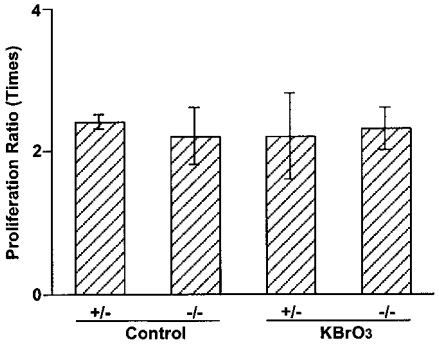

Fig. 3. Regeneration of liver in *Ogg1* mutant mice after partial hepatectomy. Partial hepatectomy was performed after $KBrO_3$ was administered at a dose of 2 grams/liter to male *gpt*/*Ogg1*$^{+/-}$ and *gpt*/*Ogg1*$^{-/-}$ mice for 12 weeks. From a measurement of liver weight, the proliferation ratio of the remnant liver during liver regeneration was calculated. Values are mean and SD.

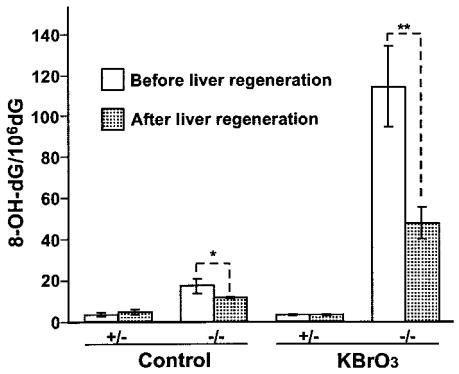

Fig. 4. The effect of cell proliferation on the levels of 8-OH-G in liver DNA. Partial hepatectomy was performed after $KBrO_3$ was administered at a dose of 2 grams/liter to male *gpt*/*Ogg1*$^{+/-}$ and *gpt*/*Ogg1*$^{-/-}$ mice for 12 weeks. The amount of 8-OH-G before (*open bars*) and after (*dotted bars*) liver regeneration was analyzed in samples that were taken at the time of partial hepatectomy and in liver samples taken 10 days after partial hepatectomy. Values are mean and SD. *, $P < 0.05$; **, $P < 0.01$.

GC→TA transversions were 35.7% before regeneration and 33.3% after. These results show that liver regeneration does not cause DNA mutations in wild-type mouse under normal conditions. GC→TA transversions in control *Ogg1*$^{-/-}$ mice (50% before regeneration and 57.1% after) were more than that of control *Ogg1*$^{+/-}$ mice. It seems that GC→TA transversion increased slightly after liver regeneration in control *Ogg1*$^{-/-}$ mice. When $KBrO_3$ was administered to *Ogg1*$^{+/-}$ and *Ogg1*$^{-/-}$ mice, the number of deletion mutants increased, although we found no deletion in *Ogg1*$^{-/-}$ mice before liver regeneration. There were no differences in mutations, other than deletions, before and after liver regeneration in $KBrO_3$-treated *Ogg1*$^{+/-}$ mice compared with control *Ogg1*$^{+/-}$ mice. In $KBrO_3$-treated *Ogg1*$^{-/-}$ mice, GC→TA transversions before and after liver regeneration were 55 and 81.6%, respectively. Cell proliferation promoted a 5.2-fold increase in mutation frequency of GC→TA (5.6×10^{-6} before regeneration and 28.9×10^{-6} after). This GC→TA mutation frequency in liver DNA from $KBrO_3$-treated *Ogg1*$^{-/-}$ mice after liver regeneration was 14.5 times that of control *Ogg1*$^{+/-}$ mice before regeneration. These results indicate that GC→TA transversion accounts for 92.1% of the mutation when 8-OH-G, generated by oxidative stress, is fixed by cell proliferation *in vivo,* and GC→TA transversion is the major mutation caused by 8-OH-G even in an animal model.

DISCUSSION

In this study, we showed that the renal carcinogen, $KBrO_3$, when administered chronically to mice, results in increased levels over time of 8-OH-G in liver DNA of *Ogg1*$^{-/-}$ mice. The amounts of 8-OH-G in liver DNA were approximately one-fifth as that of kidney DNA of *Ogg1*$^{-/-}$ mice treated with $KBrO_3$ as reported previously (29). However, the increase of 8-OH-G level in liver DNA of *Ogg1*$^{-/-}$ mice treated with $KBrO_3$ was found to be still quite significant. In addition, the accumulated DNA lesions in liver DNA did not decrease even 4 weeks after cessation of $KBrO_3$ treatment. These results indicated that OGG1 plays a major role in repair of the bulk of 8-OH-G in mouse liver. In spleen DNA, the accumulation of 8-OH-G was dependent on $KBrO_3$ treatment (data not shown). Thus, it is very likely that OGG1 is involved in repair of 8-OH-G ubiquitously in a variety of mouse tissues.

To our knowledge, this is the first report to clarify whether a cell having a high accumulation of 8-OH-G possesses the ability to proliferate. Studies in yeast have shown that DNA polymerase δ replicates only ~10% of DNA containing 8-OH-G when compared with

Table 1 *Mutation frequencies in liver cells from gpt/Ogg1$^{+/-}$ and gpt/Ogg1$^{-/-}$ mice which had undergone partial hepatectomy after treatment with KBrO$_3$ for 12 weeks*

6-TG^R and Cm^R stand for 6-TG and Cm resistant, respectively. Identical mutations found within one animal were considered the result of clonal expansion. Mutation frequencies were calculated from the number of colonies having independent mutations.

Mouse	Treatment	*Ogg1* genotype	Liver regeneration	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies		Mutation frequency ($\times 10^{-6}$)
					Total	Independent	
1	Control	+/−	Before	10.4	6	5	4.8
2	Control	+/−	Before	12.8	4	4	3.1
3	Control	+/−	Before	5.4	7	5	9.3
Mean[a]							5.7 ± 3.2
1	Control	+/−	After	8.9	3	3	3.4
2	Control	+/−	After	9.1	7	6	6.6
3	Control	+/−	After	4.1	3	3	7.4
Mean							5.8 ± 2.1
4	Control	−/−	Before	3.3	3	3	9.1
5	Control	−/−	Before	12.0	5	5	4.2
6	Control	−/−	Before	7.5	8	8	10.6
Mean							8.0 ± 3.4
4	Control	−/−	After	9.6	5	5	5.2
5	Control	−/−	After	3.5	6	6	17.2
6	Control	−/−	After	2.3	3	3	13.3
Mean							11.9 ± 6.1
7	$KBrO_3$	+/−	Before	17.7	14	8	4.5
8	$KBrO_3$	+/−	Before	17.1	10	10	5.9
9	$KBrO_3$	+/−	Before	6.8	3	3	4.4
Mean							4.9 ± 0.8
7	$KBrO_3$	+/−	After	3.8	20	5	13.2
8	$KBrO_3$	+/−	After	10.0	6	6	6.0
9	$KBrO_3$	+/−	After	4.9	3	3	6.2
Mean							8.5 ± 4.1
10	$KBrO_3$	−/−	Before	4.6	3	3	6.6
11	$KBrO_3$	−/−	Before	5.6	7	7	12.5
12	$KBrO_3$	−/−	Before	8.9	11	10	11.3
Mean							10.1 ± 3.1
10	$KBrO_3$	−/−	After	2.4	17	13	53.5
11	$KBrO_3$	−/−	After	3.7	13	10	27.3
12	$KBrO_3$	−/−	After	5.9	15	15	25.5
Mean							35.4 ± 15.7

[a] Values are mean and SD.

the replication of undamaged DNA (4). Asagoshi *et al.* (33) investigated the translesion synthesis of 8-OH-G using *E. Coli* DNA polymerase I Klenow fragment. The translesion synthesis of 8-OH-G was slower than that of G because of the inefficiency of nucleotide incorporation opposite 8-OH-G and extension past the lesion. When single-stranded DNA containing 8-OH-G was transfected to COS7 cells, the recovery yield of replicated plasmid was ~80% when compared with DNA containing G (7). A variety of genes has been identified whose transcription is initiated during the S phase of the cell cycle but whose expression is silent during the G_0-G_1 phase. Recently Le Page *et al.* (34) reported that 8-OH-G blocks transcription by RNA polymerase II. These reports led us to the idea that proliferation may be inhibited in cells having high amounts of 8-OH-G. Such cells, we envisaged, would experience cell cycle arrest or undergo apoptosis as a mechanism to prevent cancer development. Therefore, we investigated the effect of 8-OH-G on cell proliferation by partial hepatectomy of Ogg1$^{-/-}$ mice treated with $KBrO_3$. We showed that liver with a high amount of 8-OH-G can proliferate to the same extent as normal liver. Although it could be argued that rare cells with no accumulation of 8-OH-G were responsible for the proliferated tissue, we found no significant difference in the proliferation index of all liver samples examined 48 h after partial hepatectomy (data not shown). Furthermore, in comparison with liver samples taken at partial hepatectomy, after liver regeneration, significant increases in the mutation frequency were observed. The main mutation was GC→TA transversion, believed to be caused mainly by 8-OH-G.

Table 2 *Mutation spectrum of gpt gene from livers of gpt/Ogg1$^{+/-}$ and gpt/Ogg1$^{-/-}$ mice which had undergone partial hepatectomy after treatment with KBrO$_3$ for 12 weeks*

The number of colonies with independent mutations are shown. The number of total colonies are in parenthesis.

	Control				$KBrO_3$			
	Ogg1$^{+/-}$		*Ogg1*$^{-/-}$		*Ogg1*$^{+/-}$		*Ogg1*$^{-/-}$	
	Before	After	Before	After	Before	After	Before	After
Base substitution								
Transversions								
GC→TA	5	4	8	8	7	3	11	31 (38)
GC→CG	1	2	2	0	2	0	2 (3)	1
AT→TA	1	0	0	1	2	2	2	0
AT→CG	2 (3)	0	0	1	1	0	0	0
Transitions								
GC→AT	4	2	3	3	4	3 (18)	3	2
AT→GC	0	1	0	0	1 (7)	1	1	0
Deletion								
Single bp	0	1	0	1	3	2	0	2
≥2 bp	0	0	0	0	1	1	0	0
Insertion	0	0	2	0	0	0	0	0
Complex	1 (3)	2 (3)	1	0	0	2	1	2
Total	14 (17)	12 (13)	16	14	21 (27)	14 (29)	20 (21)	38 (45)

Taken together, it suggests that cells having high amounts of 8-OH-G have the ability to proliferate *in vivo*.

When partial hepatectomy was performed and liver regeneration was induced, the weight of remnant liver from the mice in all groups increased 2.2–2.4 times. After liver regeneration, the amount of 8-OH-G decreased ~50% in liver DNA from treated and nontreated *Ogg1*$^{-/-}$ mice in terms of 8-OH-G residue per guanine residue in DNA. Although the 8-OH-G level did not appear to decrease in *Ogg1*$^{+/+}$ mice, a slight dilution in the amount of 8-OH-G at steady-state levels would be difficult to distinguish from background because no technique is currently available to avoid artificial oxidation of a G base during the course of isolation of DNA (35). From these results, it is concluded that cell division dilutes the level of the DNA lesion by a factor of two, and no replication-coupled repair of 8-OH-G occurs on the parent strand. Hazra *et al.* (36, 37) discovered two new enzymes, OGG2 and NEH1, capable of removing 8-OH-G from oligonucleotides. Our results propound that these enzymes are responsible for the repair of 8-OH-G when incorporated into the nascent strand by DNA replication.

From experiments performed *in vitro,* eukaryotic DNA polymerase α and δ were shown to incorporate an A base opposite 8-OH-G during DNA synthesis on a oligodeoxynucleotide template, which contained 8-OH-G (3–5). Similarly, transfection of mammalian cells with single-stranded DNA containing 8-OH-G revealed that only GC→TA transversion occurs after replication (6, 7). However, Tan *et al.* (8) reported that 8-OH-G induced not only GC→TA but also GC→AT and GC→CG using a similar method. Kamiya *et al.* (9, 10) analyzed the mutation in foci obtained on transfection of the c-Ha-*ras* gene containing 8-OH-G to NIH3T3 cells and found GC→TA, GC→AT, and GC→CG. The fact that discrepancies exist in the tissue culture experiments compounds the need for the use of an animal model to investigate the mutation spectrum caused by 8-OH-G. Difficulties in determining the mutation spectrum generated by 8-OH-G in animals have arisen because oxidative stress reagents often produce many kinds of DNA lesions in addition to 8-OH-G. We have succeeded in analyzing the mutation spectrum caused by 8-OH-G, which accumulated in liver DNA after partial hepatectomy of *Ogg1* mutant mice treated with $KBrO_3$. Our results indicate that 8-OH-G mainly causes GC→TA transversion in an inactive gene after cell proliferation.

The activity of the OGG1 enzyme is inhibited by nitric oxide, which is associated with chronic inflammation (38). Indeed, it was reported that the 8-OH-G level in liver with chronic hepatitis was higher than that of normal liver (39). Chronic inflammation predisposes individuals to the development of carcinoma in the esophagus, gastric mucosa, pancreas, colon, and liver (40–42). From our results, it is predicted that 8-OH-G could continue to accumulate during inflammation followed by an increase in the mutation frequency after cell proliferation. We hypothesize that inactivation of the OGG1 enzyme by chronic inflammation could contribute to the risk of cancer.

It is likely that the accumulation of 8-OH-G resulting from a deficiency of the *Ogg1* gene or inactivation of the OGG1 enzyme could contribute to the initiation and promotion of cancer. For induction of cancer, the cells must be proliferated. To assess this possibility, we are planning to perform a long-term carcinogenesis test using partial hepatectomized *Ogg1* mutant mice.

ACKNOWLEDGMENTS

We thank H. Arakawa for helpful discussion and R. Sakai for DNA sequencing.

REFERENCES

1. Kasai, H., and Nishimura, S. Formation of 8-hydroxydeoxyguanosine in DNA by oxygen radicals and its biological significance. *In:* H. Sies (ed.), Oxidative Stress: Oxidants and Antioxidants, pp. 99–116. London: Academic Press, Ltd., 1991.
2. Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S., and Loeb, L. A. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G→T and A→C substitutions. J. Biol. Chem., *267:* 166–172, 1992.
3. Shibutani, S., Takeshita, M., and Grollman, A. P. Insertion of specific bases during DNA synthesis past the oxidationdamaged base 8-oxodG. Nature (Lond.), *349:* 431–434, 1991.
4. Haracska, L., Yu, S. L., Johnson, R. E., Prakash, L., and Prakash, S. Efficient and accurate replication in the presence of 7, 8-dihydro-8-oxoguanine by DNA polymerase η. Nat. Genet., *25:* 458–461, 2000.
5. Shibutani, S., and Grollman, A. P. Miscoding during DNA synthesis on damaged DNA templates catalysed by mammalian cell extracts. Cancer Lett., *83:* 315–322, 1994.
6. Moriya, M. Single-stranded shuttle phagemid for mutagenesis studies in mammalian cells: 8-oxoguanine in DNA induces targeted G·C→T·A transversions in simian kidney cells. Proc. Natl. Acad. Sci. USA, *90:* 1122–1126, 1993.
7. Le Page, F., Margot, A., Grollman, A. P., Sarasin, A., and Gentil, A. Mutagenecity of a unique 8-oxoguanine in a human Ha-*ras* sequence in mammalian cells. Carcinogenesis (Lond.), *16:* 2779–2784, 1995.
8. Tan, X., Grollman, A. P., and Shibutani, S. Comparison of the mutagenic properties of 8-oxo-7, 8-dihydro-2′-deoxyadenosine and 8-oxo-7, 8-dihydro-2′-deoxyguanosine DNA lesions in mammalian cells. Carcinogenesis (Lond.), *20:* 2287–2292, 1999.
9. Kamiya, H., Murata-Kamiya, N., Koizume, S., Inoue, H., Nishimura, S., and Ohtsuka, E. 8-Hydroxyguanine (7, 8-dihydro-8-oxoguanine) in hot spots of the c-Ha-*ras* gene: effects of sequence centexts on mutation spectra. Carcinogenesis (Lond.), *16:* 883–889, 1995.
10. Kamiya, H., Miura, K., Ishikawa, H., Inoue, H., Nishimura, S., and Ohtsuka, E. c-Ha-*ras* containing 8-hydroxyguanine at codon 12 induces point mutations at the modified and adjacent positions. Cancer Res., *52:* 3483–3485, 1992.
11. Aburatani, H., Hippo, Y., Ishida, T., Takashima, R., Matsuba, C., Kodama, T., Takao, M., Yasui, A., Yamamoto, K., Asano, M., Fukasawa, K., Yoshinari, T., Inoue, H., Ohtsuka, E., and Nishimura, S. Cloning and characterization of mammalian 8-hydroxyguanine-specific DNA glycosylase/apurinic, apyrimidinic lyase, a functional mutM homologue. Cancer Res., *57:* 2151–2156, 1997.
12. Rosenquist, T. A., Zharkov, D. O., and Grollman, A. P. Cloning and characterization of a mammalian 8-oxoguanine DNA glycosylase. Proc. Natl. Acad. Sci. USA, *94:* 7429–7434, 1997.
13. Roldan-Arjona, T., Wei, Y-F., Carter, K. C., Klungland, A., Anselmino, C., Wang, R-P., Augustus, M., and Lindahl, T. Molecular cloning and functional expression of a human cDNA encoding the antimutator enzyme 8-hydroxyguanine-DNA glycosylase. Proc. Natl. Acad. Sci. USA, *94:* 8016–8020, 1997.
14. Radicella, J. P., Dherin, C., Desmaze, C., Fox, M. S., and Boiteux, S. Cloning and characterization of *hOGG1,* a human homolog of the *OGG1* gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA, *94:* 8010–8015, 1997.
15. Lu, R., Nash, H. M., and Verdine, G. L. A mammalian DNA repair enzyme that excises oxidatively damaged guanines maps to a locus frequently lost in lung cancer. Curr. Biol., *7:* 397–407, 1997.
16. Bjorås, M., Luna, L., Johnsen, B., Hoff, E., Haug, T., Rognes, T., and Seeberg, E. Opposite base-dependent reactions of a human base excision repair enzyme on DNA containing 7, 8-dihydro-8-oxoguanine and abasic sites. EMBO J., *16:* 6314–6322, 1997.
17. Arai, K., Morishita, K., Shinmura, K., Kohno, T., Kim, S-R., Nohmi, T., Taniwaki, M., Ohwada, S., and Yokota, J. Cloning of a human homolog of the yeast *OGG1* gene that is involved in the repair of oxidative DNA damage. Oncogene, *14:* 2857–2861, 1997.
18. Zharkov, D. O., and Grollman, A. P. MutY DNA glycosylase: base release and intermediate complex formation. Biochemistry, *37:* 12384–12394, 1998.
19. Fujikawa, K., Kamiya, H., Yakushiji, H., Fujii, Y., Nakabeppu, Y., and Kasai, H. The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein. J. Biol. Chem., *274:* 18201–18205, 1999.
20. Park, Y. J., Choi, E. Y., Choi, J. Y., Park, J-G., You, H. J., and Chung, M-H. Genetic changes of *hOGG1* and the activity of oh8Gua glycosylase in colon cancer. Eur. J. Cancer, *37:* 340–346, 2001.
21. Sugimura, H., Kohno, T., Wakai, K., Nagura, K., Genka, K., Igarashi, H., Morris, B. J., Baba, S., Ohno, Y., Gao, C-M., Li, Z-Y., Wang, J-D., Takezaki, T., Tajima, K., Varga, T., Sawaguchi, T., Lum, J. K., Martinson, J. J., Tsugane, S., Iwamasa, T., Shinmura, K., and Yokota, J. hOGG1 Ser326Cys polymorphism and lung cancer susceptibility. Cancer Epidemiol. Biomark. Prev., *8:* 669–674, 1999.
22. Kohno, T., Shinmura, K., Tosaka, M., Tani, M., Kim, S-R., Sugimura, H., Nohmi, T., Kasai, H., and Yokota, J. Genetic polymorphisms and alternative splicing of the *hOGG1* gene, that is involved in the repair of 8-hydroxyguanine in damaged DNA. Oncogene, *16:* 3219–3225, 1998.
23. Hardie, L. J., Briggs, J. A., Davidson, L. A., Allan, J. M., King, R. F. G. J., Williams, G. I., and Wild, C. P. The effect of hOGG1 and glutathione peroxidase I genotypes and 3p chromosomal loss on 8-hydroxydeoxyguanosine levels in lung cancer. Carcinogenesis (Lond.), *21:* 167–172, 2000.
24. Xu, J., Zheng, S. L., Turner, A., Isaacs, S. D., Wiley, K. E., Hawkins, G. A., Chang, B-L., Bleecker, E. R., Walsh, P. C., Meyers, D. A., and Isaacs, W. B. Associations between *hOGG1* sequence variants and prostate cancer susceptibility. Cancer Res., *62:* 2253–2257, 2002.

25. Kasai, H., Nishimura, S., Kurokawa, Y., and Hayashi, Y. Oral administration of the renal carcinogen, potassium bromate, specifically produces 8-hydroxydeoxyguanosine in rat target organ DNA. Carcinogenesis (Lond.), *8:* 1959–1961, 1987.
26. Ballmaier, D., and Epe, B. Oxidative DNA damage induced by potassium bromate under cell-free conditions and in mammalian cells. Carcinogenesis (Lond.), *16:* 335–342, 1995.
27. Kurokawa, Y., Takayama, S., Konishi, Y., Hiasa, Y., Asahina, S., Takahashi, M., Maekawa, A., and Hayashi, Y. Long-term *in vivo* carcinogenicity tests of potassium bromate, sodium hypochlorite, and sodium chlorite conducted in Japan. Environ. Health Perspect., *69:* 221–235, 1986.
28. Deangelo, A. B., George, M. H., Kilburn, S. R., Moore, T. M., and Wolf, D. C. Carcinogenicity of potassium bromate administered in the drinking water to male $B6C3F_1$ mice and F344/N rats. Toxicol. Pathol., *26:* 587–594, 1998.
29. Arai, T., Kelly, V. P., Minowa, O., Noda, T., and Nishimura, S. High accumulation of oxidative DNA damage, 8-hydroxyguanine, in *Mmh/Ogg1* deficient mice by chronic oxidative stress. Carcinogenesis (Lond.), *23:* 2005–2010, 2002.
30. Minowa, O., Arai, T., Hirano, M., Monden, Y., Nakai, S., Fukuda, M., Itoh, M., Takano, H., Hippou, Y., Aburatani, H., Masumura, K., Nohmi, T., Nishimura, S., and Noda, T. *Mmh/Ogg1* gene inactivation results in accumulation of 8-hydroxyguanine in mice. Proc. Natl. Acad. Sci. USA, *97:* 4156–4161, 2000.
31. Higgins, G. M., and Anderson, R. M. Experimental pathology of the liver. Arch. Pathol., *12:* 186–202, 1931.
32. Nohmi, T., Suzuki, T., and Masumura, K. Recent advances in the protocols of transgenic mouse mutation assays. Mutat. Res., *455:* 191–215, 2000.
33. Asagoshi, K., Terato, H., Ohyama, Y., and Ide, H. Effects of a guanine-derived formamidopyrimidine lesion on DNA replication. J. Biol. Chem., *277:* 14589–14597, 2002.
34. Le Page, F., Kwoh, E. E., Avrutskaya, A., Gentil, A., Leadon, S. A., Sarasin, A., and Cooper, P. K. Transcription-coupled repair of 8-oxoguanine: requirement for XPG, TFIIH, and CSB and implications for Cockayne syndrome. Cell, *101:* 159–171, 2000.
35. Helbock, H. J., Beckman, K. B., Shigenaga, M. K., Walter, P. B., Woodall, A. A., Yeo, H. C., and Ames, B. N. DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proc. Natl. Acad. Sci. USA, *95:* 288–293, 1998.
36. Hazra, T. K., Izumi, T., Maidt, L., Floyd, R. A., and Mitra, S. The presence of two distinct 8-oxoguanine repair enzymes in human cells: their potential complementary roles in preventing mutation. Nucleic Acids Res., *26:* 5116–5122, 1998.
37. Hazra, T. K., Izumi, T., Boldogh, I., Imhoff, B., Kow, Y. W., Jaruga, P., Dizdaroglu, M., and Mitra, S. Identification and characterization of a human DNA glycosylase for repair of modified bases in oxidatively damaged DNA. Proc. Natl. Acad. Sci. USA, *99:* 3523–3528, 2002.
38. Jaiswal, M., LaRusso, N. F., Nishioka, N., Nakabeppu, Y., and Gores, G. J. Human Ogg1, a protein involved in the repair of 8-oxoguanine, is inhibited by nitric oxide. Cancer Res., *61:* 6388–6393, 2001.
39. Shimoda, R., Nagashima, M., Sakamoto, M., Yamaguchi, N., Hirohashi, S., Yokota, J., and Kasai, H. Increased formation of oxidation DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. Cancer Res., *54:* 3171–3172, 1994.
40. LaRusso, N. F. Morphology, physiology, and biochemistry of biliary epithelia. Toxicol. Pathol., *24:* 84–89, 1996.
41. Farges, O., Malassagne, B., Sebagh, M., and Bismuth, H. Primary sclerosing cholangitis: liver transplantation or biliary surgery. Surgery, *117:* 146–155, 1995.
42. Rosen, C. B., Nagorney, D. M., Wiesner, R. H., Coffey, R. J., Jr., and LaRusso, N. F. Cholangiocarcinoma complicating primary sclerosing cholangitis. Ann. Surg., *213:* 21–25, 1991.

Cell Proliferation in Liver of *Mmh/Ogg1*-deficient Mice Enhances Mutation Frequency because of the Presence of 8-Hydroxyguanine in DNA

Tsuyoshi Arai, Vincent P. Kelly, Kimiyo Komoro, et al.

Cancer Res 2003;63:4287-4292.