Mmh/Ogg1 gene inactivation results in accumulation of 8-hydroxyguanine in mice

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Edited by Philip Hanawalt, Stanford University, Stanford, CA, and approved January 7, 2000 (received for review September 21, 1999)

The major mutagenic base lesion in DNA caused by exposure to reactive oxygen species is 8-hydroxyguanine or 7,8-dihydro-8-oxoguanine (8-OH-G). Products of the human MMH/OGG1 gene are known to catalyze in vitro the reactions repairing this DNA lesion. To analyze the function of Mmh in vivo, we generated a mouse line carrying a mutant Mmh allele by targeted gene disruption. Mmh homozygous mutant mice were found to have a normally physical appearance, but to have lost nicking activity in liver extracts for substrate DNA containing 8-OH-G, exhibiting a 3-fold increased accumulation of this adduct at 9 weeks of age compared with wild-type or heterozygous mice. Further elevation to 7-fold was observed in 14-week-old animals. Substantial increase of spontaneous mutation frequencies was clearly identified in Mmh mutant mice bearing transgenic gpt genes. These results indicate that exposure of DNA to endogenous oxidative species continuously produces the mutagenic adduct 8-OH-G in mice, and Mmh plays an essential role in repair of this DNA damage.

Damage to genomic DNA is considered to be involved in aging and age-associated degenerative diseases, with 8-hydroxyguanine or 7,8-dihydro-8-oxoguanine (8-OH-G) induced by reactive oxygen species and ionizing radiation as some of the most critical mutagenic lesions. G → T transition mutations found in mammalian cells appear to be caused by mispairing of this oxidized base with A (1–3). In bacteria and Saccharomyces cerevisiae, 8-OH-G is excised by a DNA glycosylase (MutM and OGG1, respectively) with associated lyase activity for chain incision. It has remained unclear, however, whether this mammalian MMH (OGG1) has been identified and cloned (4–10). We recently mapped the clones in detail by restriction digestion, and used a crude enzyme extracts. The 21-base oligonucleotide containing 8-OH-G were synthesized with an Expedite DNA synthesizer model 8900 (PerSeptive Biosystems). The oligonucleotides were homogenized in lysis buffer (50 mM Hepes 0.1% Nonidet P-40 1 mM phenylmethylsulfonyl fluoride) and subjected to Southern blot analysis with a 1-kb PstI–SacI fragment as a probe (probe 1 in Fig. 1A). Expected sizes of hybridized bands for wild-type and mutant Mmh alleles were 15 kb and 6.8 kb, respectively. Mutant ES cells were injected into C57BL/6J blastocysts, and resulting male chimeras were mated with female C57BL/6J mice. Germ-line transmission of mutant Mmh allele to F1 mice was confirmed by Southern-blot analysis. Genotyping of F2 offspring was performed by PCR amplification using tail lysates as templates with the primers shown in Fig. 1A (M2, CTCACCTGAGTGCCCAGTGCGACA; PGK-3, CCT-GAAAGAAGATCAGCCTC; M5, CCATCCTGTG-GGCCCTGTATCTGCA).

Northern-blot analysis was carried out as described (14). Poly(A)-RNA was extracted from wild-type and Mmh mutant mouse liver by using FastTrack reagents (Invitrogen), fractionated by agarose gel electrophoresis with 1% formaldehyde, transferred to nylon membrane, and hybridized with a Mmh cDNA probe that corresponds to the sequence from exons 5–7.

AP Lyase Activity Toward 8-OH-G-Containing DNA. Mouse tissues were homogenized in lysis buffer (50 mM Hepes/250 mM NaCl/0.1% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride) with a Potter-type homogenizer and then centrifuged at 18,000 × g for 15 min. The supernatants thus obtained were used as the crude enzyme extracts. The 21-base oligonucleotide containing a single 8-OH-G (Gdh) was synthesized by the method described previously (15), and the other oligonucleotides (without 8-OH-G) were synthesized with an Expedite DNA synthesizer model 8900 (PerSeptive Biosystems). The oligonucleotides were purified by HPLC (C18-MS column, 0.46 × 25 cm, Nacalai Tesque, Kyoto) and developed with a linear acetonitrile gradient (5–30%) in 24 ml of 0.1 M triethylamine acetate (pH 7.0) at a flow rate of 1 ml/min.

Materials and Methods

Generation of Mmh-Deficient Mice. For construction of a targeting vector, we isolated five λ phage clones from a genomic library of J1 embryonic stem (ES) cells by using mouse Mmh cDNA, mapped the clones in detail by restriction digestion, and used DNA fragments of two clones for vector construction. An approximately 8.3-kb HindIII–XhoI fragment containing exons 4–7 and a 2-kb HindIII–SacI fragment containing exon 1 and the 5′ half of exon 2 were isolated and applied for construction of a targeting vector as long and short homologous sequences, respectively.

According to the procedure described previously (13), to generate mutant mice, J1 ES cells were electroporated with a targeting vector, and cells were selected in the presence of G418. To screen homologous recombinant ES cells, genomic DNA of G418-resistant clones was extracted, digested by XbaI, and subjected to Southern blot analysis with a 1-kb PstI–SacI fragment as a probe (probe 1 in Fig. 1A). Expected sizes of hybridized bands for wild-type and mutant Mmh alleles were 15 kb and 6.8 kb, respectively. Mutant ES cells were injected into C57BL/6J blastocysts, and resulting male chimeras were mated with female C57BL/6J mice. Germ-line transmission of mutant Mmh allele to F1 mice was confirmed by Southern-blot analysis. Genotyping of F2 offspring was performed by PCR amplification using tail lysates as templates with the primers shown in Fig. 1A (M2, CTCACCTGAGTGCCCAGTGCGACA; PGK-3, CCT-GAAAGAAGATCAGCCTC; M5, CCATCCTGTGGGCCCTGTATCTGCA).

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This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: 8-OH-G, 8-hydroxyguanine or 7,8-dihydro-8-oxoguanine; 8-OH-dG, 8-hydroxydeoxyguanosine; AP lyase, apurinic, apyrimidinic lyase; ES cells, embryonic stem cells; 6-TG, 6-thioguanine.

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Article published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.050404497. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.050404497.
flow rate of 1.2 ml/min. The sequences of oligonucleotides used as substrates are shown below:

- oligo 1: 5'-CAGCCAATCAGohTGCACCATCC-3'
- oligo 2: 5'-CAGCCAATCAGTGCACCATCC-3'
- oligo 3: 5'-GGATGGTGCACTGATTGGCTG-3'
- oligo 4: 5'-GGATGGTGCAGTGATTGGCTG-3'
- oligo 5: 5'-GGATGGTGCAATGATTGGCTG-3'
- oligo 6: 5'-GGATGGTGCATTGATTGGCTG-3'

An 8-OH-G-containing oligonucleotide (oligo 1) or merely G-containing oligonucleotide (oligo 2) was 5'-end labeled with [γ-32P]ATP and T4 polynucleotide kinase, and annealed with complementary oligonucleotides (oligos 3–6). Crude extracts or purified recombinant enzyme was incubated with 100 fmol of the end-labeled double-stranded oligonucleotide in 25 μl of 50 mM Tris-HCl, pH 7.5/50 mM KCl/5 mM EDTA at 37°C for 1 hr. After the incubation, labeled oligonucleotide fragments in the reaction mixture were precipitated with ethanol and isolated by electrophoresis in a 20% polyacrylamide gel containing 7 M urea. The radioactivity of the nicked products was quantified with a bioimaging analyzer (BAS2000, Fuji Photo Film, Tokyo).

Analysis of 8-OH-G in DNA with an HPLC-Electrochemical Detector (ECD) (16). Genomic DNA was extracted from 100–200 mg of tissues with a DNA Extractor WB Kit (Wako, Japan) and digested with nuclease P1 (Sigma) and acid phosphatase type XA (Sigma) at 37°C for 30 min in 100 μl of 10 mM sodium acetate solution (pH 4.5) containing 1 mM EDTA. The resulting deoxyribonucleoside mixture was treated with the ion-exchange resin Muromac (Muromachi Kagaku, Tokyo), to remove the NaI and centrifuged at 18,000 × g for 5 min. The supernatant was filtered with Microcon 10 (Amicon) and analyzed with an HPLC-ECD system: pump, L-6000 (Hitachi); UV detector, L-4000 (Hitachi); ECD, Coulochem (ESA); column, Beckman Ultrasphere ODS (0.46 × 25 cm); eluent, 10 mM NaH₂PO₄ buffer containing 8% methanol; flow rate, 1 ml/min. The amounts of deoxyguanosine (dG) and 8-hydroxydeoxyguanosine (8-OH-dG) in the DNA samples were measured with UV at 290 nm and ECD (guard cell, 1 V, detector I, 1 V, detector II, 1.5 V), respectively. Ten micrograms of dG (Sigma) and 100 pg of 8-OH-dG (Sigma) were injected as standards. The ratio of 8-OH-dG to dG in the DNA sample was determined from the peak areas of the standards.

All measurements of AP lyase activity and 8-OH-G amount in this study were performed on a blind-test basis.

Spontaneous Mutation Assay in Vivo. gpt transgenic mice carrying ecogpt genes for detection of mutations introduced in vivo were used...
to estimate spontaneous mutation frequency in Mmh homozgyous mutant mice compared with wild-type mice, gpt transgenic male mice of C57BL6 background were mated with Mmh F0 chimera to obtain F1 (gpt/Mmh+/−) mice. Mmh homozygous mutant (Mmh−/−) and wild-type (Mmh+/+) mice hemizygous for the gpt transgene were obtained by mating F1 (Mmh+/−) males with F1 (gpt/Mmh+/+) females. The copy number of gpt genes serving as targets of mutagenesis was estimated as approximately 80 per hemizygous allele in autosomes. High molecular weight DNAs of mice carrying the gpt transgene were extracted from liver according to the method of Suzuki et al. (17) and used for rescuing λ phage containing a linear plasmid. The gpt gene is included in this plasmid, and it was excised from the λ phage vector in host Escherichia coli expressing Cre recombinase to give a circularized form that eventually confers sensitivity to 6-thioguanine (6-TG). To rescue λ phages from the DNA, an in vitro packaging reaction was performed with TransPack extracts (Stratagene). Four micrograms of genomic DNA from an Mmh mutant mouse carrying the gpt transgene was used for each packaging reaction, and the resultant lysate was suspended in 500 μl of SM buffer (50 mM Tris-HCl, pH 7.5/10 mM MgSO4/100 mM NaCl/0.01% gelatin). All aliquots of the 500-μl phage suspension were infected into E. coli strain YG6020 expressing Cre recombinase to determine mutant frequencies for the gpt reporter gene. Infected cells were poured on plates containing 1.5% agar, 1x M9 salts, 1% glycerol, 2 mM MgSO4, 0.1 mM CaCl2, 5 μg/ml thiamin, 50 μg/ml proline, 50 μg/ml leucine, 50 μg/ml isoleucine, 25 μg/ml chloramphenicol, and 25 μg/ml 6-TG (18). For estimating the total rescue efficiencies of phages from mouse genomic DNA, diluted suspensions of infected cells were poured on plates containing chloramphenicol without 6-TG. Plates were incubated for 3 days at 37°C for selection of colonies harboring the plasmids carrying mutated gpt genes. To confirm the results of 6-TG selection, surviving colonies were replated on fresh 6-TG plates and incubated for another 3 days. The gpt mutant frequency was calculated as M/(W × d) where M, W, and d stand for total number of mutant colonies on a 6-TG plate, number of colonies on a plate without 6-TG, and the fold dilution, respectively.

To analyze the mutation spectrum, the entire gpt gene of each mutant was amplified by the PCR procedure with a primer pair as follows: Gpt-1seq: TACCACTTTATCCCGCGTCAGG; Gpt-2seq: ACAGGGTTTCGCTCAGGTTG. Obtained PCR products were purified and sequenced directly with an ABI Prism 210 DNA Sequencer (Applied Biosystems).

**Results**

**Genetic Inactivation of the Mmh Gene in Mice.** Using homologous recombination in ES cells, we obtained two independent ES clones (cl 132 and cl 165) carrying mutant alleles of Mmh, in which the 3' half of exon 2 and the entire sequence of exon 3 were replaced by the pgk-neo gene cassette (Fig. 1A). Chimeric mice were generated and germ-line transmission of mutant Mmh alleles to F1 offspring was confirmed for chimeric mice generated from both ES clones (Fig. 1B). To produce F2 progeny for the analysis, interbreeding of F1 heterozygotes originating from the cl 165 ES cell was performed. The results described in this paper were obtained from the analysis of the resultant F2 mice. Genotype analysis of 86 F2 offspring was performed by PCR (Fig. 1C): cl 22 were wild type (25%), 41 were heterozygous (48%), and 23 were homozygous mutants (27%), consistent with the ratio expected from Mendelian inheritance. Mmh expression was not detected in the liver of the Mmh homozygous mutant by Northern-blot analysis (Fig. 1D). Clearly the Mmh gene product does not play an essential role in embryonic development, and both homozygous and heterozygous mutants appeared healthy with no obvious differences from wild-type littermates in survival and gross appearance until 50 weeks old. No tumor formation has been observed thus far. Both male and female homozygotes proved fertile, and F3 homozygous offspring obtained by homozygous intercrosses were also found to be healthy.

**AP Lyase Activity in Mmh Mutant Liver Cells.** Because mammalian MMH/OGG1 products have been shown to have glycosylase/AP

**Fig. 2.** AP lyase activity in mouse liver crude extracts. A 5’-end-labeled oligonucleotide containing an 8-OH-G hybridized with the complementary oligonucleotide was incubated with the crude extracts, and then nicked products produced were analyzed by 20% PAGE. (A) Representative AP lyase assay: lane 1, 1 ng of purified recombinant human MMH type 1a protein as a positive control; lane 2, the lysis buffer as a negative control; lanes 3 and 4, 5 and 20 μg of crude extract of liver from an Mmh wild-type mouse; lanes 5 and 6, 5 and 20 μg of crude extract of liver from an Mmh heterozygous mutant mouse; lanes 7 and 8, 5 and 20 μg of crude extract of liver from an Mmh homozygous mutant mouse. S, substrate oligonucleotide; N, nicked product. (B) Quantification of the nicked products by analyzing radioactivity with a bioimaging analyzer BAS2000. Numbers of mice used are indicated under the genotype. Results are mean and SD. *, P < 0.0003; **, P < 0.0001 (Fisher statistical analysis)
lyase activity that may specifically excise 8-OH-G in DNA, AP lyase activity in liver extracts of Mmh mutant mice was analyzed. Nicking activity toward substrate DNA with 8-OH-G-C pairs was measured by titrating the radioactivity of nicked products on gels. As shown in Fig. 2, the results showed a clear decrease of activity consistent with the gene dosage; 16 fmol/25 μg of protein for the wild type, 7.0 for heterozygous mutants, and 0.36 for homozygous mutants. Although not totally zero when compared with negative control (Figs. 2A and 3), the residual activity of unknown origin in homozygotes was at most less than 5% of that for wild-type mice. The substrate specificity of nicking activity in liver extracts was also determined (Fig. 3). In wild-type mice it was in the following order: 8-OH-G/C > 8-OH-G/T > 8-OH-G/G > 8-OH-G/A > G/C, identical to findings for recombinant human MMH type 1a protein (rhMMH type 1a or 1a) or lysis buffer (−) were incubated with the substrates listed above and then the nicked products were analyzed by 20% PAGE.

Fig. 3. Substrate specificity of AP lyase activity in crude liver extracts of Mmh wild-type, Mmh heterozygous mutant, and Mmh homozygous mutant mice. The 8-OH-G- or G-containing oligonucleotide was labeled and annealed with one of the four complementary oligonucleotides having C, A, T, or G opposite the 8-OH-G or G. The crude liver extracts, purified recombinant human MMH type 1a protein (rhMMH type 1a or 1a) or lysis buffer (−) were incubated with the substrates listed above and then the nicked products were analyzed by 20% PAGE.

Fig. 4. Accumulation of 8-OH-G in DNA of Mmh-deficient mice. (A) Electrochemical chromatograms of 8-OH-dG in Mmh mutant mouse liver genomic DNAs. The HPLC patterns were traced by an electrochemical (EC) detector as described in Materials and Methods. Patterns are for 8-OH-dG level, in 1, a wild-type mouse; 2, an Mmh heterozygous mutant mouse; 3, an Mmh homozygous mutant mouse at 9 weeks of age; and 4, an Mmh homozygous mutant mouse at 14 weeks of age. Arrows indicate 8-OH-dG peaks, the areas of those for homozygotes being markedly increased. (B) The measured amounts of 8-OH-G in liver tissues from Mmh wild-type, heterozygous mutant, and homozygous mutant mice at 9 and 14 weeks of age are summarized. Values are mean ± SD (n = 3). The 8-OH-G levels in Mmh homozygous mutant mice were significantly higher than those in Mmh wild-type mice at both 9 and 14 weeks of age at P < 0.0001 (Fisher’s test). The 8-OH-G levels in 14-week-old homozygotes were significantly higher than those in 9-week-old Mmh homozygous mutant mice at P < 0.0001.

Minowa et al. PNAS | April 11, 2000 | vol. 97 | no. 8 | 4159
gotes. From these observations we conclude that major repair of 8-OH-G is carried by the Mmh gene product, at least in mouse liver cells.

**Accumulation of 8-OH-G in DNA of Mmh Mutant Mice.** To analyze the effects of Mmh deficiency on oxidative DNA lesion accumulation in mice, the amount of 8-OH-G in genomic DNA was measured by a HPLC–electrochemical detection method, which is highly selective, with a sensitivity at the fmol level (Fig. 4). In DNA of wild-type mice the amount was measured to be 1.0–2.0 in 10^6 dG, in good agreement with our previous report (20). A significant increase was observed in the liver DNA of Mmh homozygous mutants (Fig. 4B): at 9 weeks of age, the level of 8-OH-G in liver DNA was 6.6 in 10^6 dG, about 4.2-fold the wild-type value, and further increase to 12.0 in 10^6 dG was observed in 14-week-old animals. This indicated accumulation of 5.4 8-OH-G in 10^6 dG within 5 weeks, or approximately 3,900 8-OH-Gs every week per diploid DNA of liver cells that are deficient in the Mmh gene product. Although heterozygotes possessed only half the AP lyase activity of wild-type mice, this seemed to be enough to process the usual 8-OH-G load, since no statistically significant elevation was apparent.

**Increased Mutation Frequencies in Mmh Mutants.** Mmh homozygous mutant (Mmh−/−) and wild-type (Mmh+/+) mice hemizygous for the gpt transgene, serving as a target of mutagenesis, were established to assess spontaneous mutation frequencies in the mutant mice. Liver DNA of six Mmh homozygous mutants 16–20 weeks old and six wild-type mice of the same age, all carrying the gpt gene, were used independently for the assay. The number of gpt mutant colonies (6-TG-resistant and chloramphenicol-resistant), the total number of colonies plated (chloramphenicol-resistant), and the calculated mutation frequencies for each mouse are shown in Table 1. Consistent with the previous report (18), the mutation frequencies for gpt/Mmh−/− transgenic mice were under 1 × 10^{-3}, although the values fluctuated among these six mice. On the other hand, in liver DNAs obtained from six Mmh homozygous mutants, the mutation frequencies tended to increase and, on average, 2.3-fold elevation of the frequencies was noted. This increase was considered to be statistically significant (Fisher’s test, P < 0.05). To analyze the spectrum of mutations introduced spontaneously in vivo, plasmids were recovered from gpt mutant colonies and the ORFs of the gpt gene were sequenced. Mutations thus found were all single-base substitutions. In Mmh-deficient mice, 26 out of a total of 46 base substitutions (57%) were G → T or C → A transversions, and the rest were 15 G → A transitions and 5 T → A transversions. In the wild type, 8 G → T or C → A transversions out of 23 single-base-pair substitutions (35%) were found. This mutation spectrum implies that more than 70% of the increase in mutations in Mmh homozygotes was accounted for by G → T or C → A transversions. Because 8-OH-G has been shown to mispair with A, resulting in G → T transversions (1–3), these results suggest that the majority of increased mutation in vivo was caused by the 8-OH-G formation in Mmh-deficient mice.

**Discussion**

The results of this study provide evidence of an essential role of Mmh in the mouse in repair of 8-OH-G, the major mutagenic base lesion in DNA caused by exposure to reactive oxygen species. Naturally these species may be derived from both exogenous and endogenous sources. Our findings indicate that oxidative adducts in mouse liver cells are constantly produced by endogenous oxygen species of endogenous origin, resulting in age-dependent accumulation of 8-OH-G in the absence of the Mmh gene product in mice. Mmh deficiency further caused increase of spontaneous mutation frequency in vivo largely because of the mispairing of A with 8-OH-G in DNA.

Previous reports have suggested the presence of two distinct enzymes to repair 8-OH-G in human cells (12, 21). A mutation of the Msh2 gene, one of the mismatch repair genes, has also been found to cause accumulation of 8-OH-G in mouse ES cells (23). Although there is a clear discrepancy between these results and ours with respect to basal levels of 8-OH-G in DNA from wild-type controls, DeWeese et al. (22) reported that ES cells from Msh2−/− mice accumulated much larger amounts of 8-OH-G in DNA compared with Msh2+/+ mice, either with or without low-level radiation treatment in tissue culture system. The results of our study described here, however, clearly indicate that the Mmh gene product may be solely responsible for repair of 8-OH-G in liver cells of mice. Bessho et al. (23) previously showed that mammalian N-methylpurine-DNA glycosylase releases 8-OH-G from DNA in vitro as well as in vivo when transfected into E. coli. The excision of 8-OH-G from synthetic DNA in vitro by a human nucleotide excision repair system was also reported by Reardon et al. (24). On the basis of our results,
it is likely that none of these enzymatic systems plays a major role in repair of 8-OH-G in the mouse liver.

In this study, significantly increased levels of 8-OH-G in Mmh-deficient mice were observed. Levels of 8-OH-G detected in liver DNA from wild-type mice are approximately 1.5 8-OH-G per 10^6 dG in the system we used in the present study. It is possible that this is an over-estimation because of artificial generation of 8-OH-G during the course of isolation of DNA. In that case, deletion of Mmh must have exerted a devastating effect on increase of 8-OH-G in cellular DNA. A relatively low spontaneous mutation rate and absence of tumors does not appear consistent with the marked accumulation of potentially miscoding 8-OH-G residues in the genome, as observed here for Mmh-deficient liver tissues. There are several possible explanations of this discrepancy. First, it may be merely that fixation of mutations is limited because only a small fraction of adult liver cells are proliferating. Second, it could be that the human homologue of the MutY DNA glycosylase provides repair of the opposite strand. It can excise A misincorporated opposite 8-OH-G in dividing cells. Such removal would result in possible reversion to a correct C. Third, there could be another complementary repair of 8-OH-G by the nucleotide excision-repair (NER) pathway. The NER system was shown to repair of potentially miscoding 8-OH-G residues in the genome, as observed here for Mmh-deficient liver tissues. There are several possible explanations of this discrepancy. First, it may be merely that fixation of mutations is limited because only a small fraction of cell turnover must have exerted a devastating effect on large increase of 8-OH-G in DNA and the observed relatively small increase in spontaneous mutations in livers of Mmh-deficient mice. Further study on the appropriate synthetic mutant mice is required to clarify this functional overlapping of the Mmh gene with other repair systems in mammals.

During revision of this paper, Klungland et al. (25) reported on independently obtained Mmh/Ogg1-deficient mice. They noted accumulation of 8-OH-G and increase of spontaneous mutation frequency in Mmh/Ogg1-deficient mice, consistent with our results.

We thank Eiko Ohtsuka for valuable reagents. We also express our appreciation to Hitomi Yamanaka, Yoshinobu Sugitani, Akiko Kawakami, Shiko Ito, and Hiromi Takahashi for their expert technical support and to Toshiyuki Kobayashi for valuable discussions. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Sports, Science and Culture of Japan (O.M. and T.N.).