The Mammalian Mismatch Repair Pathway Removes DNA 8-oxodGMP Incorporated from the Oxidized dNTP Pool

Claudia Colussi,1,5 Eleonora Parlanti,1,5 Paolo Degan,2,5 Gabriele Aquilina,1 Deborah Barnes,3 Peter Macpherson,3 Peter Karran,3 Marco Crescenzi,1 Eugenia Dogliotti,1 and Margherita Bignami1,4
1Laboratory of Comparative Toxicology and Ecotoxicology
Istituto Superiore di Sanita’
Rome
2Istituto Nazionale per la Ricerca sul Cancro
Genova
Italy
3Cancer Research UK
London Research Institute
Clare Hall Laboratories
South Mimms
United Kingdom

Summary
Mismatch repair (MMR) corrects replication errors. It requires the MSH2, MSH6, MLH1, and PMS2 proteins which comprise the MutSα and MutLα heterodimers [1]. Inactivation of MSH2 or MLH1 in human tumors greatly increases spontaneous mutation rates [2]. Oxidation produces many detrimental DNA alterations against which cells deploy multiple protective strategies [3]. The Ogg-1 DNA glycosylase initiates base excision repair (BER) of 8-oxoguanine (8-oxoG) from 8-oxoG:C pairs [4]. The Myh DNA glycosylase removes mismatched cytosines incorporated opposite 8-oxoG during replication [5]. Subsequent BER generates 8-oxoG:C pairs, a substrate for excision by Ogg-1. MTH1—an 8-oxoGTPase which eliminates 8-oxoGTP from the dNTP pool—affords additional protection by minimizing 8-oxodGMP incorporation during replication [6]. Here we show that the dNTP pool is, nevertheless, an important source of DNA 8-oxoG and that MMR provides supplementary protection by excising incorporated 8-oxodGMP. Incorporated 8-oxodGMP contributes significantly to the mutator phenotype of MMR-deficient cells. Thus, although BER of 8-oxoG is independent of Msh2, both steady-state and H₂O₂-induced DNA 8-oxoG levels are higher in Msh2-defective cells than in their repair-proficient counterparts. Increased expression of MTH1 in MMR-defective cells significantly reduces steady-state and H₂O₂-induced DNA 8-oxoG levels. This reduction dramatically diminishes the spontaneous mutation rate of Msh2−/− MEFs.

Results

MMR and Accumulation of DNA 8-oxoG
Msh2 status influences the steady-state and oxidant-induced level of DNA 8-oxoG. DNA of untreated msh2−/− mouse embryo fibroblasts (MEFs) contained approximately 2-fold more oxidized guanines than DNA from msh2+/+ MEFs. The average values were 0.68 and 0.34 8-oxoG per 10⁶ guanines for msh2−/− and wild-type cells, respectively (p < 0.0001, Student’s t test for paired samples). There was evidence of a gene dosage effect as the levels of DNA 8-oxoG in msh2−/− heterozygous cells were intermediate (0.47 per 10⁶ guanines) between those of wild-type and homozygous cells (Figure 1A). Msh2 status also influenced the amount of DNA 8-oxoG introduced by exposure to an oxidizing agent (Figure 1B). Following treatment of wild-type cells with 10 or 20 mM H₂O₂, the level of 8-oxoG increased to 1.7 and 3.3 per 10⁶ guanines, respectively. By comparison, the same treatment produced 3-fold more DNA 8-oxoG in msh2−/− MEFs. H₂O₂-treatment of msh2−/− cells resulted in intermediate levels of the oxidized purine (data not shown).

The effect of Msh2 deficiency on the steady-state level of DNA 8-oxoG is not confined to MEFs. Colo5 is an Msh2-defective variant of the MMR-proficient Colo26 mouse colon tumor cell line [7]. The DNA of repair-defective Colo5 cells contained almost 4-fold more 8-oxoG than DNA of the parental Colo26 cells (33.9 versus 8.8 per 10⁶ guanines) (Figure 1C). These values are more than 20-fold higher than the corresponding values for the MEFs, possibly because these cells are derived from a murine colon tumor. A normal copy of human chromosome 2, which contains the hMSH2 gene, corrects the MMR defect of Colo5. It also reduced the steady-state level of DNA 8-oxoG by almost 50% (Figure 1B). The value of 18.3 per 10⁶ guanines in Colo5 + Ch2 DNA, which is somewhat higher than the corresponding one for untreated Colo26 cells, is compatible with an msh2 gene dosage effect and expression of a single copy of hMSH2.

Increased levels of DNA 8-oxoG in Msh2-defective cells could reflect either a MMR defect or a MMR-independent role for Msh2. We tested these possibilities in two ways. First, we examined the level of 8-oxoG in DNA from hMLH1-deficient cells. Abrogation of the downstream hMLH1 MMR function in a set of closely related human A2780 ovarian carcinoma cell lines was also associated with increases in both the steady-state and induced levels of DNA 8-oxoG (Figure 2). A2780 Clone 1 cells are defective in MMR because their hMLH1 gene is silenced by promoter hypermethylation [8, 9]. The resting level of DNA 8-oxoG in A2780 Clone 1 cells was 4-fold higher (1.4 versus 0.3 8-oxoG per 10⁶ guanines) than in the MMR-proficient parental A2780 cells, which resembled the msh2−/− MEFs in this regard. A transfected hMLH1 cDNA corrects the repair defect in A2780 Clone 1. hMLH1 expression decreased the steady-state 8-oxoG level to 0.8 per 10⁶ guanines (Figure 2A). hMLH1 deficiency also affected oxidant-induced DNA 8-oxoG levels, and there was approximately 2-fold more 8-oxoG in the DNA of H₂O₂-treated A2780 Clone 1 cells than in DNA of their MMR-proficient A2780 parents. This difference was a consequence of the MMR defect in A2780 Clone 1 cells, and the H₂O₂-induced levels of DNA
8-oxoG were closely similar in the hMLH1 transfectants and parental A2780 cells (Figure 2B).

### Base Excision Repair of 8-oxoG

In the second approach, we examined whether Msh2 could act as a modifier of the Ogg1 DNA glycosylase that removes 8-oxoG from resting DNA. Ogg1 activity in cell extracts was assayed by measuring incision of a 30-mer duplex oligonucleotide containing a single 8-oxoG:C base pair. Ogg1 activity was similar in extracts of MEFs representing all three msh2 genotypes (Figure 3A). Surprisingly, despite their high levels of DNA 8-oxoG, Colo26 cell extracts contained significantly more Ogg1 activity than extracts of MEFs. Colo26, Colo5, and Colo5 + ch 2 extracts all incised the 8-oxoG duplex approximately 10-fold more efficiently than the MEF extracts (Figure 3B). There were no detectable differences among the Msh2-proficient and -defective variants of Colo26 in this regard. It is unclear why elevated levels of DNA 8-oxoG coexist with a high Ogg1 activity in these mouse tumor cells. It may reflect unusual kinetics of the Ogg1-mediated BER. Alternatively, it may indicate that the DNA 8-oxoG in these cells is in a form that is not recognized by Ogg1.

The capacity of MEF extracts to complete BER paralleled their levels of Ogg1 activity. We observed no significant differences in the rate, extent, or patch size of BER of a circular duplex containing a single 8-oxoG residue [10] by extracts of msh2+/+ or msh2−/− MEFs (data not shown). These direct biochemical determinations indicate that, at least in resting DNA, Msh2 is unlikely to be a significant influence on BER of 8-oxoG:C pairs.

### Combined Ogg1 and Msh2 Mutations

A complementary genetic approach using MEFs from msh2−/−/ogg1−/− animals provided additional evidence for the independence of Ogg1 from Msh2. The steady-state levels of 8-oxoG in DNA of ogg1−/− and msh2−/− MEFs were similar despite their slightly different genetic backgrounds (Figure 4A). DNA from ogg1−/− MEFs contained 1.7-fold more 8-oxoG than DNA from ogg1+/+ cells, in agreement with published data [11]. The effects of Msh2 and Ogg1 inactivation on the steady-state DNA 8-oxoG level were additive, and DNA from msh2−/−/ogg1−/− MEFs contained 4-fold more 8-oxoG than wild-type MEF DNA (Figure 4A).

Inactivation of ogg1 also affected the amount of 8-oxoG in DNA after H2O2 treatment. This effect was also additive with that of msh2 inactivation (Figure 4B). Treatment with 20 mM H2O2 increased the level of DNA 8-oxoG to 1.9 per 10^6 guanines in ogg1−/− MEFs and to 4.3 in msh2−/− MEFs. The value of 6.0 8-oxoG per 10^6 guanines in DNA of ogg1−/−/msh2−/− cells was approximately the sum of the values of both single knockout cells.

Thus, the Msh2 MMR protein and the Ogg1 DNA glycosylase both affect the steady-state level of DNA 8-oxoG. They also modify the extent to which the oxidized purine accumulates in DNA after brief treatment with an oxidizing agent. In both cases, Msh2 and Ogg1 act independently, and their effects are additive. The demonstration that hMLH1 has a comparable effect on DNA 8-oxoG levels strongly suggests that the MMR pathway provides significant protection against the accumulation of the oxidized purine.

### Incorporation of 8-oxoG: Overexpression of hMTH1

We investigated whether MMR might be counteracting the incorporation of 8-oxodGMP from the oxidized dNTP pool. The cDNA that encodes the human MTH1 8-oxodGTPase [12] was introduced into Msh2-proficient or -defective cells by transfection. MTH1 expression was analyzed by Western blotting of extracts of individual clones. Expression of the transfected hMTH1 was readily detectable, whereas extracts from nontransfected cells contained only low levels of protein recognized by the anti-hMTH1 antibody (Figure 5A and data not shown). hMTH1 expression was quantified by measuring dGTPase activity in the extracts. The low dGTPase levels of untransfected cells were increased up to 10-fold in the transfectants. A 5- to 10-fold increase...
in MTH1 activity significantly reduced the steady-state levels of DNA 8-oxoG in Colo26 and Colo5 (Figure 5B). The effect of hMTH1 expression was most pronounced in the Msh2-defective Colo5 cells in which the steady-state 8-oxoG levels were highest.

A similar trend was observed in hMTH1-transfected MEFs (Figure 5C). Because the steady-state levels of DNA 8-oxoG were much lower in these cells, the extent of reduction by hMTH1 was more modest. In five independent experiments, a 10-fold increased expression of hMTH significantly reduced DNA 8-oxoG levels in msh2−/− MEFs (0.71 versus 0.50; p < 0.01, Student’s t test for paired samples). A more pronounced reduction was observed in a single msh2−/− MEF clone in which hMTH1 expression was 5-fold higher. This high-level hMTH1 expression reduced the steady-state level of DNA 8-oxoG to around or below that of msh2+/+ MEFs (0.25 per 10⁶ guanines). The level of MTH1 expression in msh2+/+ MEFs was more modest (Figure 5C). In this case, the difference in 8-oxoG levels between hMTH1-expressing msh2+/+ cells and their nontransfected counterparts did not reach statistical significance (0.41 versus 0.37; Student’s t test for paired samples; not significant). This suggests that MTH activity may not be limiting in MMR-proficient MEFs, although we note that, in addition to a lower level of MTH1 expression, these measurements of DNA 8-oxoG are close to the detection limits for HPLC/EC.

The modest level of hMTH1 expression did significantly diminish H₂O₂-induced levels of DNA 8-oxoG in both msh2+/+ and msh2−/− MEFs (Figure 5D). In msh2+/+ cells treated with 20 mM H₂O₂, hMTH1 expression reduced DNA 8-oxoG from 3.1 to 2.2 per 10⁶ guanines. The effect of hMTH was more pronounced in H₂O₂-treated msh2−/− cells in which a somewhat higher expression of MTH1 reduced DNA 8-oxoG levels to almost half—from 6.3 to 3.8 per 10⁶ guanines.
Discussion

Our findings confirm the contribution of both Msh2 [13] and Ogg1 [11] to maintaining low steady-state and oxidant-induced levels of DNA 8-oxoG. Biochemical and genetic approaches concur that Msh2 and Ogg-1 act independently in this, and our results effectively exclude a significant role for Msh2 in Ogg-1-mediated BER. The demonstration that both the hMLH1 and Msh2 MMR factors influence DNA 8-oxoG levels strongly suggests that the MMR pathway contributes significantly to the exclusion or removal of 8-oxoG from DNA. Preferential removal of 8-oxoG from the transcribed strand of transfected DNA molecules is also partially independent of the standard assay. (A) Western blot analysis of hMTH1. Extracts of Colo26 and Colo5 were separated on 7.5% SDS polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad), and probed overnight with anti-hMTH1 antibody followed by the appropriate secondary antibody. Blots were developed using the ECL detection reagents (Amersham). The arrow indicates the position of the 18 KDa hMTH1 protein. (B) The effect of transfected hMLH1 on DNA 8-oxoG in Colo26 and Colo5. Steady-state DNA 8-oxoG levels were measured in nontransfected (unfilled bars) or hMTH1-transfected (filled bars) Colo26 and Colo5 cells. The relative levels of MTH activity are shown above the appropriate bar. The values for untransfected Colo26 and Colo5 are 0.3 and 0.4, respectively. The values of 8-oxoG are the means of two independent determinations. (C) Steady-state levels of DNA 8-oxoG in msh2+/− and msh2−−. Untransfected (unfilled bars) or hMTH1-transfected msh2+/− MEFs (filled/hatched bars) (mean, n = 5). MTH activity values shown are relative to untransfected msh2+/− and msh2−− MEFs. These values were 0.45 and 0.4, respectively. (D) DNA 8-oxoG induced by 60 min exposure to 20 mM H2O2 in msh2+/− and msh2−− MEFs. Values are means (n = 3).
Figure 6. The Four Exclusion Pathways for DNA 8-oxoG

Left panel (top): OGG-1 excises 8-oxoG from resting DNA. Oxidation of DNA guanine by reactive oxygen species (ROS) forms promutagenic 8-oxoG:C pairs in nonreplicating DNA. These are corrected by base excision repair initiated by the OGG-1 DNA glycosylase. Left panel (bottom): MYH counteracts mutation at replicated 8-oxoG lesions. During replication, dAMP is inserted opposite unrepaired 8-oxoG. This promutagenic base pairing is counteracted by the MYH DNA glycosylase, which removes the incorporated adenine base from the A:8-oxoG pair. This initiates a base excision repair event in which the insertion of C opposite the lesion generates a substrate for subsequent 8-oxoG removal by Ogg-1. Right panel (top): MTH1 provides a supplementary line of defense. Direct oxidation of the dGTP pool by ROS generates 8-oxodGTP. This damaged DNA precursor is eliminated by MTH. This prevents the incorporation of promutagenic 8-oxodGMP into DNA. Under conditions of severe oxidative stress, MTH levels might become limiting, leading to the extensive incorporation of 8-oxodGMP. Right panel (bottom): Mismatch repair (MMR) carries out additional surveillance for DNA 8-oxoG. MMR involving the MSH2 and MLH1 proteins excises 8-oxodGMP incorporated during replication. This removal involves the long patch mismatch repair pathway initiated by the MutSa and MutLa MMR complexes.

Ogg1 [14]. The role, if any, of mammalian MMR in this transcription coupled repair (TCR) is not well understood. Our in vitro assays mimic BER of 8-oxoG in resting DNA and therefore do not address TCR. Since only 5% of the genome is actively transcribed, we consider unlikely that the 100% increase in the level of DNA 8-oxoG in MMR-defective cells reflects the participation of MMR factors in TCR.

The key finding that a transfected hMTH1 brings about a significant reduction of DNA 8-oxoG levels in both MEFs and mouse tumor cells provides the first direct evidence that the oxidized dNTP pool is a significant source of DNA 8-oxoG in these cells. The ability of hMTH1 to decrease the level of both steady-state and oxidant-induced DNA 8-oxoG indicates that endogenous oxidation of the dNTP pool is an important source of DNA damage and that the dNTP pool is a significant target for exogenous oxidative damage. Taken together, our observations indicate that MMR excises incorporated 8-oxodGMP from newly synthesized daughter DNA. Yeast provides a precedent for MMR at 8-oxoG-containing base pairs generated during replication [15, 16]. In that organism, MMR appears to fulfill the role of the E. coli MutY DNA glycosylase for which no homolog has been identified in the yeast genome [16]. MutY and yeast MMR both initiate correction of the A-containing daughter strand at the 8-oxoG:A pairs formed frequently during replication. To do this, the yeast MutSr recognizes and binds 8-oxoG:A base pairs. This property is shared by the human hMutSr mismatch recognition complex [17, 18], which also selectively recognizes 8-oxoG:T and 8-oxoG:G but not the Ogg1 substrate 8-oxoG:C [17]. Unlike yeast, mammalian cells, including the MEF and Colo26 cells used in this study (data not shown), express MYH, the mammalian homolog of the MutY DNA glycosylase. Our findings—in particular the independence of Ogg-1 and Msh2 in keeping DNA free of 8-oxoG—are not consistent with a simple functional redundancy between MYH and MMR. The most likely substrate for intervention of MMR is the 8-oxoG:A mispairs formed when 8-oxoG is incorporated into daughter DNA opposite a template A. In view of the substrate preferences of the hMutSr complex, we cannot exclude intervention at the more infrequent 8-oxoG:T or 8-oxoG:G mispairs, however. We conclude that DNA 8-oxoG is excluded from newly synthesized DNA by a combination of MTH-mediated 8-oxodGTP hydrolysis and excision by MMR (Figure 6). Compromising or saturating the latter function results in the accumulation of DNA 8-oxoG. MMR therefore joins MTH, Ogg-1-, and MYH- (and possibly Ogg-2-
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References

24. Fiumicino, S., Martineilli, S., Colussi, C., Aquilina, G., Leonetti,


