Current Topics

Biological and Pharmaceutical Aspects of Nucleic Acids Chemistry

Mutagenicities of 8-Hydroxyguanine and 2-Hydroxyadenine Produced by Reactive Oxygen Species

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Oligodeoxyribonucleotides containing 8-hydroxyguanine and 2-hydroxyadenine, purine lesions produced in cells by reactive oxygen species, were synthesized and inserted into vector DNAs to introduce each lesion at a pre-determined site. The manipulated DNAs were transfected into living cells, and the mutants induced by each DNA lesion were collected and analyzed. In addition, the mutations induced by damaged DNA precursors with the two oxidized purine bases were studied by the use of chemically synthesized nucleoside triphosphates. In this review article, the author summarizes the mutagenic potentials of the two oxidized purine bases, by focusing on experiments examined by the author and his collaborators.

Key words 2-hydroxyadenine; 8-hydroxyguanine; DNA lesion; damaged nucleotide; reactive oxygen specy; mutation

Oncogene activation and tumor suppressor gene inactivation are caused by mutations. The formation of damaged DNA and damaged DNA precursors (2'-deoxyribonucleoside 5'-triphosphates) is an important source of mutations. Many approaches have been used to study the relationship between damaged nucleic acids and mutations. One of the most effective approaches is the introduction of a chemically synthesized DNA lesion or damaged DNA precursor into living cells. This method has been used to elucidate the mutagenic properties of various DNA lesions in the past fifteen years, and was aided by developments in nucleic acids chemistry and molecular biology.

Reactive oxygen species (ROS) form an important group of endogenous mutagens, which oxidize DNA and its precursors. ROS are involved in mutagenesis, carcinogenesis, aging, and neurodegeneration,^{1–3)} due to their endogenous production in addition to their formation by many environmental mutagens and carcinogens. When dA, dG, dATP, dGTP, and DNA are treated with a Fenton-type reagent *in vitro*, 8-hydroxyguanine (8-OH-Gua, 7,8-dihydro-8-oxoguanine) and 2-hydroxyadenine (2-OH-Ade) are formed (Table 1).^{4,5)} The author and his collaborators examined the mutagenicities of these damaged purine bases produced by ROS, in both DNA and the nucleotide pool, using synthetic oligodeoxyribonucleotides (ODNs) and nucleotides. In this review, the author wishes to summarize the data on the mutagenic properties of 8-OH-Gua and 2-OH-Ade. First, the mutational properties of 8-OH-Gua in DNA, and next, the mutagenicity of 2-OH-Ade in DNA will be described. Finally, the mutations induced by the oxidized nucleotides 8-hydroxy-dGTP (8-OH-dGTP) and 2-hydroxy-dATP (2-OH-dATP) will be summarized.

1. SUBSTITUTION MUTATIONS INDUCED BY 8-OH-Gua IN MAMMALIAN CELLS

8-OH-Gua is now recognized as one of the most important DNA lesions, due to its prevalence in DNA and its mutagenicity in bacterial and mammalian cells, as described

Table 1. Yields of 8-OH-Gua and 2-OH-Ade Formed *in Vitro*

Materials	Treatment	Yields (%) ^{a)}	
		8-OH-Gua	2-OH-Ade
dA/dG	Fe ²⁺ -EDTA	2.6 (1.0)	4.8 (1.8)
	Fe ²⁺ -NTA	0.7 (1.0)	0.4 (0.6)
dATP/dGTP	Fe ²⁺ -EDTA	3.7 (1.0)	0.7 (0.2)
ds DNA	Fe ²⁺ -EDTA	0.4 (1.0)	0.01 (0.03)

a) Relative yields are shown in parentheses.

below. Additionally, this compound is a useful marker for DNA oxidation,^{6,7)} since it can be sensitively detected by an HPLC system equipped with an electrochemical detector. Elution of the 8-OH-Gua content in DNA has been reported in many experiments with cultured cells and animals that were exposed to 'oxidative stress'.^{6,7)}

To determine the type of mutation induced by 8-OH-Gua in a mammalian system, synthetic c-Ha-*ras* proto-oncogenes containing an 8-OH-Gua residue in hotspots (codons 12, 61) were constructed and transfected into mouse NIH3T3 cells.^{8,9)} Most base substitution mutations that result in the conversion of an amino acid residue in these c-Ha-*ras* gene hotspots can activate the gene, and thus, they display focus-forming activity upon transfection into NIH3T3 cells.^{10,11)} 8-OH-Gua residues were incorporated into the first and second positions of codon 12 in the sense strand (5'-GGC-3') and into the first position of codon 61 in the antisense strand (5'-CAA-3', antisense strand 5'-TTG-3') of the gene during ODN synthesis.^{8,9)} Transfection of this gene significantly increased the number of transformed foci, indicating that the introduced 8-OH-Gua caused base substitutions. The relative transforming efficiencies of the c-Ha-*ras* genes with 8-OH-Gua in codon 12 or in codon 61 are about 1–2% of that of an activated c-Ha-*ras* gene (Val-12 or Asp-12, and Lys-61 or His-61). This value is interpreted as the mutation frequency (MF) of 8-OH-Gua in double-stranded (ds) DNA in mammalian cells (Table 2).

The c-Ha-*ras* gene present in these foci was analyzed by

Table 2. Mutational Properties of 8-OH-Gua in NIH3T3 Cells

Sequence ^{a)} (5'→3')	MF (%) ^{b)}	Mutations detected			
		G→T	G→A	G→C	Others
GG*C ^{c)}	ca. 1.4	8	3	1	2 ^{d)}
GG*C ^{e)}	ca. 1.7	22	8	1	11 ^{f)}
G*GC ^{e)}	ca. 0.6	14	1	0	0
TTG* ^{e)}	ca. 1.0	35	0 ^{g)}	0 ^{g)}	5 ^{h)}

a) G* represents 8-OH-Gua. b) MF is calculated based on the focus formation relative to that of activated c-Ha-ras genes. c) From ref. 8. d) Two mutants contained a mutation at a flanking site. e) From ref. 9. f) Eleven mutants contained a mutation at a flanking site. g) Mutations at this position do not activate the c-Ha-ras gene. Mutants may fail to be selected due to lack of focus-formation. h) Five mutants contained a mutation at a flanking site.

the polymerase chain reaction-restriction enzyme method.^{8,9)} Interestingly, the sequence analysis revealed that the 8-OH-Gua residues at the first positions of codons 12 and 61 (5'-(8-OH-Gua)GC-3' and 5'-TT(8-OH-Gua)-3', respectively) almost exclusively induced mutations to T at the modified sites (Table 2). On the other hand, this DNA lesion at the second position of codon 12 (5'-G(8-OH-Gua)C-3') induced a G→A transition in addition to a G→T transversion. Mutations in 5'-flanking sites were also elicited when 8-OH-Gua was present at the second position of codon 12 or the first position of codon 61. These results suggest that dATP is misincorporated opposite 8-OH-Gua, and dTTP is also misinserted in a sequence-dependent manner, during DNA replication in NIH3T3 cells. Moreover, the presence of 8-OH-Gua may affect the nucleotide incorporation opposite the 5'-flanking site.

Others have examined the mutagenicity of 8-OH-Gua in single-stranded (ss) vectors in mammalian cells.^{12–14)} They reported that 8-OH-Gua elicited G→T transversions, with an MF of 4–7% in simian COS-7 cells. In addition to G→T transversions, G→A, G→C and semitargeted mutations at the 5'-flanking positions were detected. Taken together with our data shown above, the mutational properties of 8-OH-Gua in mammalian cells may depend upon the sequence context, the host cells, and/or whether ss/ds vectors were used for the experiments.

The mutagenic potential of 8-OH-Gua was also investigated with *Escherichia coli* as a host. 8-OH-Gua in ss or ds DNA induced G→T transversions.^{15–18)} The MF of 8-OH-Gua in ds DNA is 0.1–1%, in agreement with our results.^{16,18)} However, almost no mutations other than G→T transversions were found. Thus, the mutational properties of 8-OH-Gua slightly differ in bacterial and mammalian cells.

Synthetic ODNs containing 8-OH-Gua near the 5'-end, mimicking the nucleotide incorporation step, were designed to examine their thermodynamic stabilities.¹⁹⁾ We introduced the 8-OH-Gua into the sites corresponding to the first and second positions of codon 12 of the c-Ha-ras gene, to compare the thermodynamic stabilities of the base pairs involving 8-OH-Gua and the mutation spectra obtained with the modified c-Ha-ras genes. In duplexes with various bases at the 3'-end, located opposite 8-OH-Gua, remarkably high stability (a small ΔG^0 value) was found in the case of the 8-OH-Gua:T pair in the 5'-G(8-OH-Gua)C-3' sequence, while no stabilization was observed with the 8-OH-Gua:T pair in the other context. These thermodynamic stabilities may be re-

lated to the mutation spectra described above (the G→A mutation in the 5'-G(8-OH-Gua)C-3' sequence, Table 2). On the other hand, the thermodynamic stabilities did not correlate with the mutation spectra, when duplexes with a central base pair involving 8-OH-Gua were examined.¹⁹⁾

In contrast, the results from *in vitro* DNA synthesis experiments, with the same ODNs as those used in the NIH3T3 study, did not agree with the above results. Mammalian DNA polymerase (pol) α incorporated dATP, but not dTTP, opposite 8-OH-Gua, even in the DNA template with the 5'-G(8-OH-Gua)C-3' sequence.^{20,21)} Thus, the G→A transition in the 5'-G(8-OH-Gua)C-3' sequence, found in the NIH3T3 study, may be due to the misincorporation of dTTP by DNA polymerase(s) δ and/or ϵ , or other components of the DNA replication machinery. Alternatively, lesion-bypass DNA polymerase(s) may be involved in the mutation process. Interestingly, Nishimura and his collaborators recently reported that human pol η caused misincorporation opposite G at the 5'-flanking site of 8-OH-Gua in the 5'-G(8-OH-Gua)C-3' sequence.²²⁾ This may be related to the observed 5'-flanking mutations in NIH3T3 cells (Table 2).

2. MUTATIONS INDUCED BY 2-OH-Ade IN LIVING CELLS

2-OH-Ade is an oxidized form of adenine. The yields of 2-OH-Ade are similar to those of 8-OH-Gua in the monomeric form, although its formation in DNA is less efficient upon treatment with a Fenton-type reagent *in vitro* (Table 1).^{4,5)} Thus, it is likely that the incorporation of 2-OH-dATP by DNA polymerases from the nucleotide pool is the major pathway for the formation of 2-OH-Ade in DNA. The formation of 2-OH-Ade in DNA has been assessed by GC-MS. 2-OH-Ade was formed in cellular DNA upon H₂O₂ treatment of human lymphoblast cells, upon Fe-NTA treatment of rat hepatocytes, and upon metal-H₂O₂ treatment of isolated human chromatin.^{23–25)} 2-OH-Ade was also generated after nickel acetate was injected into pregnant rats.²⁶⁾ In addition, γ -ray irradiation of cultured human cells and mice induced 2-OH-Ade accumulation in cellular DNA.^{27,28)} Moreover, 2-OH-Ade is present in human cancerous tissues.²⁹⁾ The reported amounts of 2-OH-Ade were similar to those of 8-OH-Gua. Moreover, as shown below, 2-OH-Ade possesses a mutation-inducibility similar to that of 8-OH-Gua in living cells. Thus, 2-OH-Ade seems to be another important form of DNA damage produced by ROS.

To analyze the nucleotide misincorporation induced by 2-OH-Ade in DNA, twelve synthetic ODNs containing 2-OH-Ade with different 5'- and 3'-flanking bases were used as DNA templates for *in vitro* DNA synthesis.^{30,31)} DNA polymerases inserted various nucleotides opposite 2-OH-Ade in the presence of a single dNTP, depending upon the sequence contexts and the DNA polymerases used. The two mammalian DNA polymerases (α and β) incorporated dCTP most frequently among the 'incorrect' nucleotides, while dGTP was inserted more efficiently than dCTP and dATP by the *E. coli* Klenow fragment. Interestingly, the three DNA polymerases inserted dATP opposite 2-OH-Ade in the 5'-T(2-OH-Ade)A-3' sequence, as observed using two different templates containing the 5'-T(2-OH-Ade)A-3' sequence. This conclusion was confirmed by a comparison of the full-

length products obtained by *in vitro* DNA synthesis in the presence of the four nucleotides.^{30,31)}

The mutations induced by 2-OH-Ade in *E. coli* and mammalian cells were analyzed using a ds shuttle vector (Table 3).^{32,33)} The oxidized adenine was incorporated into 5'-G(2-OH-Ade)C-3' and 5'-T(2-OH-Ade)A-3' sequences, based on the results of the *in vitro* experiments. In addition, the differences in the mutagenic properties of 2-OH-Ade in the lagging and leading template strands were studied. ODNs containing 2-OH-Ade were chemically synthesized, and were ligated into a shuttle vector plasmid together with the complementary ODN. The constructed DNAs were then transfected into *E. coli* and simian COS-7 cells.

The frequency and the spectrum of the induced mutations depended on the sequence contexts and the lagging/leading synthesis. The MFs observed in these experiments were about 0.1–0.8% (Table 3), which are comparable to the MFs of 8-OH-Gua in ds DNA in *E. coli* and mammalian cells.^{8,9,16,18)} Therefore, 2-OH-Ade in DNA is as mutagenic as 8-OH-Gua. The variations between the lagging and leading strand syntheses may be due in part to the differences in the DNA polymerases and the other accessory proteins involved in DNA replication.³⁴⁾

In *E. coli*, the mutations observed most frequently were –1 deletions (ΔA) at both positions during lagging strand synthesis (Table 3). On the other hand, we observed that 2-OH-Ade induced A→G and A→T substitutions during leading strand synthesis. These results indicate that 2-OH-Ade residues in DNA induce substitution and deletion mutations in *E. coli*. The A→G and A→T substitutions elicited by 2-OH-Ade suggest the incorporation of dCTP and dATP, respectively. In addition, the observed –1 deletions at the 5'-G(2-OH-Ade)C-3' and 5'-T(2-OH-Ade)A-3' sites can be explained by the incorporation of dCTP and dATP, respectively, opposite the 2-OH-Ade residue, and the subsequent formation of loop-out intermediates.³²⁾ Thus, these results suggest the formation of 2-OH-Ade:C and 2-OH-Ade:A pairs in bacterial cells. A→C transversion mutations were also found, suggesting that dGTP was also misincorporated opposite 2-OH-Ade in bacterial cells.

Similar, but different, results were obtained with simian COS-7 cells. When 2-OH-Ade in the 5'-G(2-OH-Ade)C-3' sequence was introduced, the mutation induced most frequently during the lagging strand synthesis was a –1 deletion (Table 3). A→G transitions and A→T transversions as well as –1 deletions were also induced during leading strand synthesis. In the case of 2-OH-Ade in the 5'-T(2-OH-Ade)A-3' sequence, the same number of colonies contained the ΔA mutation and targeted A→G transitions during the lagging strand synthesis. In contrast, A→G transitions and A→T transversions, but no deletion mutants, were elicited by 2-OH-Ade in the 5'-T(2-OH-Ade)A-3' sequence during the leading strand synthesis. As described above, A→G transitions in the 5'-G(2-OH-Ade)C-3' sequence, A→T transversions in the 5'-T(2-OH-Ade)A-3' sequence, and ΔA mutations appear to be elicited by the expected misincorporation of a nucleotide. A→T mutations in the 5'-G(2-OH-Ade)C-3' sequence and A→G mutations in the 5'-T(2-OH-Ade)A-3' sequence were also detected. These results suggest the formation of 2-OH-Ade:C and 2-OH-Ade:A pairs in COS-7 cells. In contrast to the *E. coli* results, no A→C transversions

Table 3. Mutational Properties of 2-OH-Ade in *E. coli* and COS-7 Cells

Sequence ^{a)} (5'→3')	Host	Lagging/ leading	MF (%)	Mutations detected			
				A→G	A→T	A→C	ΔA
GA*C	<i>E. coli</i>	Lagging	0.77	6	0	1	20
TA*A	<i>E. coli</i>	Lagging	0.05	2	0	2	10
GA*C	<i>E. coli</i>	Leading	0.20	14	0	0	5
TA*A	<i>E. coli</i>	Leading	0.07	3	9	1	4
GA*C	COS-7	Lagging	0.60	3	0	0	21
TA*A	COS-7	Lagging	0.09	6	1	0	6
GA*C	COS-7	Leading	0.10	4	3	0	7
TA*A	COS-7	Leading	0.11	10	5	0	0

a) A* represents 2-OH-Ade.

were observed in mammalian cells.

Synthetic ODNs containing 2-OH-Ade near the 5'-end, mimicking the nucleotide incorporation step, were designed to examine their thermodynamic stabilities.³⁵⁾ The order of stability was T>G>C>>A in the 5'-G(2-OH-Ade)C-3' sequence and T>A>C>G in the 5'-T(2-OH-Ade)A-3' sequence. Since T, G, and C, and T and A are the nucleotides incorporated *in vitro* opposite 2-OH-Ade in the 5'-G(2-OH-Ade)C-3' and 5'-T(2-OH-Ade)A-3' sequences, respectively, (see above)^{30,31)} these results agree with the miscoding properties of 2-OH-Ade residues. A stable 2-OH-Ade:A pair was formed for the 5'-T(2-OH-Ade)A-3' sequence, but not for the 5'-G(2-OH-Ade)C-3' sequence, in molecular modeling.³⁵⁾

3. MUTATIONS INDUCED BY OXIDIZED PURINE NUCLEOTIDES

The fact that an *E. coli* mutator gene (*mutT*) encodes a hydrolyzing enzyme for 8-OH-dGTP indicates the incorporation of this oxidized nucleotide by DNA polymerases and its importance as a source of mutations.³⁶⁾ The contributions toward the accumulation of 8-OH-Gua in DNA are shared almost equally by the incorporation 8-OH-dGTP from the nucleotide pool and the direct oxidation of G bases in DNA.³⁷⁾ In addition, the fact that the damaged nucleotides incorporated into bacterial cells elicit chromosomal gene mutations (as described below)^{38,39)} provides direct evidence that damaged DNA precursors act as mutagens.

It was reported that 8-OH-dGTP was incorporated opposite A and C *in vitro*.^{36,40,41)} The ratio of the incorporation of 8-OH-dGTP opposite A to that opposite C depends upon the DNA pol. Misincorporation of 8-OH-dGTP opposite G or T has not been reported. On the other hand, the misincorporation mode of 2-OH-dATP is DNA pol-specific. We incubated synthetic ODN templates with DNA polymerases in the presence of 2-OH-dATP. *E. coli* DNA pol III α (catalytic) subunit and human DNA pol η inserted 2-OH-dATP opposite T and G.^{42,43)} In contrast, mammalian pol α incorporated 2-OH-dATP opposite T and C.⁴⁾ Interestingly, the misincorporation of nucleotides opposite 2-OH-Ade in DNA templates is also DNA pol-specific (see above). We also examined the misincorporation of these oxidized purine nucleotides in a gap-filling reaction by the *E. coli* DNA pol III holoenzyme.⁴⁴⁾ 8-OH-dGTP induced A→C mutations, as also reported previously by others.¹⁶⁾ 2-OH-dATP elicits G→T transversions almost exclusively. The mutagenicity of 2-OH-dATP was higher

Table 4. Mutation Spectra of 8-OH-dGTP and 2-OH-dATP in *E. coli*

Nucleotide	Mutations detected					
	G:C→A:T	A:T→G:C	G:C→T:A	G:C→C:G	A:T→T:A	A:T→C:G
8-OH-dGTP	0	1	2	1	3	61
2-OH-dATP	2	1	37	1	2	1

than that of 8-OH-dGTP.

We developed a new strategy to evaluate the mutagenicity of a damaged DNA precursor in *E. coli*. 8-OH-dGTP and 2-OH-dATP were introduced into competent *E. coli*, and mutants with alterations in the chromosomal *lacI* gene were analyzed.³⁸⁾ Both damaged nucleotides induced *lacI* gene mutations in a dose-dependent manner, while unmodified dATP and dGTP did not elicit the mutations. The addition of 50 nmol of 8-OH-dGTP and 2-OH-dATP into an *E. coli* suspension induced 12- and nine-fold more substitutions than the control case, respectively. The 8-OH-dGTP induced A:T→C:G transversions, and the 2-OH-dATP elicited G:C→T:A transversions (Table 4). These results indicate that the two oxidatively damaged nucleotides are mutagenic *in vivo*.

Information about the mutagenicities of damaged nucleotides, such as 8-OH-dGTP and 2-OH-dATP, in living mammalian cells would be quite interesting, but no reports on them have been published. Instead, the SV40 origin-dependent *in vitro* replication system, a good model for replication in living cells, was used. Pavlov *et al.* studied the effects of 8-OH-dGTP on the fidelity of replication conducted by a HeLa cell extract.⁴⁵⁾ The replicated DNA was transfected into *E. coli* and induced A:T→C:G mutations. We recently examined the mutagenicity of 2-OH-dATP in mammalian cells, using this experimental system.⁴⁶⁾ 2-OH-dATP is mutagenic during *in vitro* replication with a HeLa extract, and this nucleotide induces substitution and deletion mutations. Among the substitutions, G:C→A:T transitions, including tandem (CC→TT) mutations, and G:C→T:A transversions were mainly elicited.

The induction of A→C (A:T→C:G) transversions induced by 8-OH-dGTP can be explained by its incorporation opposite A, and the subsequent insertion of dCTP opposite the incorporated 8-OH-Gua residue during the next round of replication. In the case of 2-OH-dATP, the occurrence of the G→T (G:C→T:A) transversion and G:C→A:T transition mutations can be explained by its incorporation opposite G and C, respectively, and the following insertion of dTTP opposite the incorporated 2-OH-Ade residue during the next round of replication. These explanations agree with results obtained by *in vitro* DNA synthesis experiments.^{4,36,40–43)} As discussed in the previous paper,⁴⁷⁾ the DNA pol-specific mispairing properties of 2-OH-dATP may be derived from the presence of the hydrophobicity-dependent enol-keto equilibrium of 2-OH-Ade, and from the possibility of adopting the *syn* conformation.

4. CONCLUSION

Synthetic ODNs containing an oxidized purine base were successfully employed to elucidate their mutagenic potentials

in living bacterial and mammalian cells. DNA precursors with these damaged bases were introduced into living *E. coli*, which revealed their mutational properties. These types of studies, together with investigations on the formation of damaged nucleic acids, DNA repair, and nucleotide pool sanitization, are biologically important,⁴⁸⁾ and will provide a molecular basis and excellent screening systems for the development of new drugs that suppress mutagenesis, carcinogenesis, aging, and neurodegeneration. In addition, damaged mutagenic DNA precursors as shown here, may be used as anticancer and antiviral drugs, with excellent delivery systems.

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