Neighboring base damage induced by permanganate oxidation of 8-oxoguanine in DNA

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ABSTRACT

We found that single-stranded DNA oligomers containing a 7,8-dihydro-8-oxoguanine (8-oxo-G) residue have high reactivity toward KMnO₄; the oxidation of 8-oxo-G induces damage to the neighboring nucleotide residues. This paper describes the novel reaction in detail, including experiments that demonstrate the mechanism involved in the induction of DNA damage. The results using DNAs of various base compositions indicated that damaged G, T and C (but not A) sites caused strand scissions after hot piperidine treatment and that the damage around the 8-oxo-G occurred at G sites in both single and double strands with high frequency. The latter substrates were less sensitive to damage. Further, kinetic studies of the KMnO₄ reaction of single-stranded oligomers suggested that the reactivity of the DNA bases at the site 5'-adjacent to the 8-oxo-G was in the order G > A > T, C. This preference correlates with the electron donating abilities of the bases. In addition, we found that the DNA damage at the G site, which was connected with the 8-oxo-G by a long abasic chain, was inhibited in the above order by the addition of dG, dA or dC. On the other hand, the damage reactions proceeded even after the addition of scavengers for active oxygen species. This study suggests the involvement of a redox process in the unique DNA damage initiated by the oxidation of the 8-oxo-G.

INTRODUCTION

One of the most common forms of oxidative damage to DNA in vivo is the generation of 7,8-dihydro-8-oxoguanine (8-oxo-G; also referred to as 8-hydroxyguanine, Fig. 1) from guanine. This damage may lead to mutations (1,2), which play a significant role in cancer (1) and aging (3), as well as some other human diseases (4,5). 8-Oxo-G is a better electron donor than any of the natural nucleosides (6), which allows its presence in DNA to be selectively detected with an electrochemical detector (7). The high reactivity of 8-oxo-G towards oxidation in vitro suggests that it may be also be sensitive to in vivo oxidation. Our investigation with permanganate was designed to explore some of the potential consequences of further oxidation. We found that an 8-oxo-G residue in DNA is more sensitive to oxidation by permanganate than thymine and the other common bases (8). The sensitivity of 8-oxo-G, as the nucleoside or in DNA, to singlet oxygen (9,10) or triplet-excited carbonyl (11,12) has been reported, but there have been no reports on the fate of the nucleotide residues next to or near the 8-oxo-G after oxidation of the modified base. Here we report full details of the damage to the bases of neighboring nucleotides that occurs when the single 8-oxo-G residue in single- or double-stranded oligodeoxynucleotides is oxidized with permanganate. Also, we describe several experiments, including the use of oligomers with abasic site analogue(s) or hexa(ethylene glycol) linker(s) (Fig. 1), to examine the nature of the damage around the 8-oxo-G. The data suggest that a redox (electron transfer) reaction participates in the induction of the DNA damage.

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Table 1. Sequences, cleavage positions and percentages of 5′- or 3′-end-labeled single strands

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>Cleavage yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5′GGCGCGCGTG3′</td>
<td>4.8 73(29) (47)</td>
</tr>
<tr>
<td>2</td>
<td>5′GGCGCGCGTG3′</td>
<td>53 32(80) (4.8)</td>
</tr>
<tr>
<td>3</td>
<td>5′GGCGCGCGTG3′</td>
<td>2.7 13 26 28(58) (9.8) (3.4)</td>
</tr>
<tr>
<td>4</td>
<td>5′GGCCAGCGTG3′</td>
<td>7.9 63(57) (8.0) (10) (4.1)</td>
</tr>
<tr>
<td>5</td>
<td>5′GGCCTTCGCGTG3′</td>
<td>4.3 20 47(62) (11) (9.7)</td>
</tr>
<tr>
<td>6</td>
<td>5′GGCGCGCGTG3′</td>
<td>15 54</td>
</tr>
<tr>
<td>7</td>
<td>5′ATGACGGAAATAT3′</td>
<td>16 44 18(64)</td>
</tr>
<tr>
<td>8</td>
<td>5′TTTCGTTCTT3′</td>
<td>15 18 66 28(56) (2.7) (0.4)</td>
</tr>
<tr>
<td>9</td>
<td>5′TTTTGTTTTT3′</td>
<td>2.0 9.0 7.7 18 20(39) (5.5) (2.5) (2.4) (1.3)</td>
</tr>
<tr>
<td>3b</td>
<td>5′GGCGCGCGTG3′</td>
<td>2.7 13 26 32</td>
</tr>
<tr>
<td>8b</td>
<td>5′TTTCGTTCTT3′</td>
<td>14 19 5.9 26</td>
</tr>
</tbody>
</table>

aAll reactions were performed under the conditions described in Materials and Methods. G and bold letters refer to 8-oxo-G and cleavage sites, respectively. Cleavage yields in parentheses were obtained from the experiments with 3′-end-labeled oligomers. Cleavage percentages correspond to those for sites with 5′ to 3′ shown at the left and the underlined percentages are for 8-oxo-G residues. Cleavage yields at the Ts of the 8-oxo-G-containing strands after the KMnO₄ oxidation were corrected by subtraction of the cleavage yields at the Ts of the control strands. Also, the cleavage yields (4–8%) at the 8-oxo-G site upon hot piperidine treatment alone (see Text) were subtracted from those for the 8-oxo-G-containing strands after the oxidation.

bThe cleavage yields were obtained from the experiment with an oxygen-free buffered solution of 5′-end-labeled 3′ or 7.

RESULTS

Damage of single-stranded DNA detected by strand cleavage analysis

Various single-stranded oligodeoxynucleotides containing a single 8-oxo-G residue in the middle of a 12mer (13mer for 9) were prepared (Table 1) according to the reported method (13). We first examined the susceptibility of the 8-oxo-G to permanganate oxidation using oligonucleotides 1 and 2 (5′-end-labeled), which correspond to a part of the human c-Ha-ras gene with a G-rich sequence and a modified codon 12. Since the results showed that the reaction occurred not only at the 8-oxo-G site but also, unexpectedly, at the 5′-upstream or 5′-next G position in the strands (Table 1), we next investigated the base- and region-specificities of this reaction. Strand 3 is an analog of 2 with the substitution of a cytosine base (C) 5′-adjacent to the G-(8-oxo-G) site with G. Strands 4, 5 and 6 are also analogs of 2 and differ only by a single base [adenine (A), thymine (T) and C, respectively] 5′-adjacent to the 8-oxo-G. Strand 7 corresponds to the sequence of codons 1–4 of the c-Ha-ras gene, which is A, T-rich as compared with 1–6. Strands 8 and 9 have pyrimidine sequences (T-tracts for 9) on both sides of the 8-oxo-G.

Oligonucleotides 1–9 and their control strands with G instead of 8-oxo-G were 5′- or 3′-end-labeled with 32P, treated with KMnO₄, and subjected to hot alkaline treatment followed by denaturing 20% polyacrylamide gel electrophoresis (PAGE). Autoradiograms of the reaction products from 3 and 8 are shown in Figure 2A and B. Lanes 3 and 12 show the piperidine-induced scission without oxidation at the 8-oxo-G position (14,15) of strands 3 and 8, which can be compared with the results for their control strands (no scission, lanes 7 and 17). The minor cleavage at the 8-oxo-G by the direct piperidine treatment may be due to the decomposition of the 8-oxo-G by aerobic oxidation under basic conditions, because the cleavage was inhibited in the presence of 2-mercaptoethanol (15 and our independent result). Lanes 4, 13 and 14 represent the permanganate-induced damage at the 8-oxo-G and its neighboring G and T residues, which can be detected by piperidine-induced scission; no cleavage was observed without piperidine treatment (lanes 5 and 15). The damage of the control strands was found only as specific damage at T sites (lanes 8 and 19) (16,17). It should be noted that KMnO₄ is a classic reagent for the oxidation of double bonds in organic molecules and the primary products from the oxidation of pyrimidine bases are 5,6-dihydroxy-5,6-dihydro derivatives (18). The reactivity of the common bases in DNA toward permanganate oxidation (37°C and pH 9 for 19 h) occurs in the order T > C > G, and A is inert toward the oxidation (19). Figure 2C shows the time-course of the reaction of 3, which indicates early induction and an increase in the damage of the neighboring nucleotide residues. Table 1 represents the damaged positions of 1–9, as analyzed by the strand scission and the cleavage yields. According to the above observations, the cleavage yields at the 8-oxo-G and T positions were normalized.
Figure 2. Autoradiograms of reaction products from 5′-end-labeled 3 (A) and 8 (B), and their control strands after denaturing 20% PAGE. The KMnO₄ oxidation of 3, 8 and the control strands, followed by the strand cleavage analysis of the products, was performed under the conditions described in Materials and Methods. Lanes 1, 3–5: 3; lanes 2, 7–9: the control strand for 3; lanes 11–15: 8; lanes 6, 10: Maxam–Gilbert A+G lanes for 3 and the control strand, respectively. Lanes 13 and 18, and 14 and 19 have 23 µM and 120 µM permanganate concentrations, respectively. (C) A time course of the strand cleavage of 3 after oxidation and the following piperidine treatment. The numbers above each lane refer to the reaction time (min). G* designates G or 8-oxo-G.

The 8-oxo-G residue was highly reactive to the oxidation, as expected, and the other damage was observed near the 8-oxo-G (at both the 5′ and 3′ sides of the 8-oxo-G). The results indicated that the reactivity of the common base residues was apparently in the order G > T > C. For example, the G residue 5′-adjacent to the 8-oxo-G in strand 2 was cleaved efficiently (53% yield) after oxidation and piperidine treatment, whereas the corresponding T site in 5 and the corresponding C site in 6 gave cleavage yields of 20 and 15%, respectively. In addition, it appears that as the position of G (or T) moves further away from the 8-oxo-G, the cleavage at the G (or T) position becomes less efficient. The tendency can clearly be seen in the case of the T-dominant oligonucleotide 9. On the other hand, a comparison between the data for 9 and its control strand with G instead of 8-oxo-G indicates that their patterns of strand cleavage were quite different and, in the latter case, the Ts of the middle part of the strand were rather insensitive to the oxidation (20%): the intrinsic T cleavage (%) of a part of the 5′-end-labeled control strand (5′-TTT1T2T3T4TTT-3′): T1, 12; T2, 10; T3, 8.3; T4, 4.6.

The results described here demonstrate that this novel damage is initiated by the oxidative modification of 8-oxo-G (also see the next section) and that it has base-selectivity.

Damage of single-stranded DNA detected by nucleoside composition analysis

Although the strand cleavage analysis showed the presence of damage to G, T and C, the damage to adenine residues could not be elucidated by this analysis. To test the possibility that the damaged A site is inert to piperidine-induced strand cleavage and that for other bases there might also be types of damage not leading to strand scission, the nucleoside composition of the oxidized oligomers was analyzed after the permanganate oxidation. The analysis was carried out by enzymic digestion of the oxidation products of 4, 7, 8 and their control strands with snake venom phosphodiesterase and alkaline phosphatase, followed by detection of the resulting nucleosides by reversed-phase HPLC. The analysis showed that the 8-oxo-2′-deoxyguanosine (8-oxo-dG) completely disappeared and that a large amount (~70%) of the 2′-deoxyadenosine (dA) also disappeared after the oxidation of 4 (Fig. 3), although dA was intact in the control experiments with the control strand. On the other hand, the reduction in the amounts of dG, dC and dT did not exceed the degree of their damage, as determined by the strand scission analyses of 4, 7 and 8. For example, 21% of the entire amount of dG was found to be damaged after the oxidation and this was comparable to the degree (30%) of damaged G elucidated by the strand cleavage analysis. In addition, new products detectable with a UV (254 nm) monitor were not observed for 4, 7 and 8 under the same HPLC conditions. These results, as well as the strand cleavage analysis, indicated that the damage of the common nucleosides, except for the dA residues, in 8-oxo-G-containing single-stranded DNAs would exclusively result in strand cleavage by the piperidine treatment. Also, the data that the strand scissions of 5′-end-labeled oligomers were observed only at the 5′-side region of the 8-oxo-G (for the 3′-end-labeled, at the 5′-side region) (Table 1) mean that the 8-oxo-G residue was always damaged whenever the neighboring residues were damaged.
reaction within the DNA chain. If a redox mechanism, which may contribute to the damage migration, is involved in the reaction, the entire reactivity of the 8-oxo-G-containing oligomers should depend on the electron donating activities of the DNA bases (G > A > T > C) (22,23) around the 8-oxo-G residue [redox potentials of DNA bases are as follows: guanine, +1.29; adenine, +1.39; thymine, +1.49; cytosine, +1.64 (in V versus saturated calomel electrode) (22)]. In other words, the reactivities of the oligomers would be higher when Gs exist near the 8-oxo-G, than when pyrimidines are located near the modified base. In order to test this hypothesis, we determined the pseudo-first-order rate constants ($k_{obs}$) for the oxidation of seven oligomers 1–6 and 8 (Table 2).

Table 2. Pseudo-first-order rate constants for the KMnO$_4$ oxidation of 8-oxo-G-containing single strands$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>$k_{obs}$ (x$10^{-3}$/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5'GCCCGGCGGTTG3'</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>5'GCCCGGCGGTTG3'</td>
<td>3.3</td>
</tr>
<tr>
<td>1</td>
<td>5'GCCCGGCGGTTG3'</td>
<td>3.2</td>
</tr>
<tr>
<td>4</td>
<td>5'GCCCGGCGGTTG3'</td>
<td>&lt; 2.4</td>
</tr>
<tr>
<td>5</td>
<td>5'GCCCGGCGGTTG3'</td>
<td>1.8</td>
</tr>
<tr>
<td>6</td>
<td>5'GCCCGGCGGTTG3'</td>
<td>1.7</td>
</tr>
<tr>
<td>8</td>
<td>5'TTTTTTGTGCCTT3'</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$^a$Pseudo-first-order rate constants were determined on the basis of the time course of the decrease of the substrate oligomers. The procedure for this determination is described in Materials and Methods.

The results indicate that the rate constants may depend on the electron donating activities of the bases 5'-flanking the 8-oxo-G. Strand 3, which has a continuous sequence of Gs 5'-adjacent to the 8-oxo-G was most reactive ($k_{obs}$ = 3.8 $\times$ 10$^{-3}$/s) among the seven substrates, whereas strand 8, which contains only pyrimidines except for the 8-oxo-G, had the smallest constant ($k_{obs}$ = 1.1 $\times$ 10$^{-3}$/s).

It should be noted that the present method for the determination of the rate constants, in principle, is not dependent on whether the damage sites are cleaved or not. The 8-oxo-G residue has been always damaged whenever the damage is induced at the neighboring residues, and the damaged 8-oxo-G site is certainly cleaved so as to lead to the consumption of the substrates. Thus, the results from Table 2 indicate that the reactivity of the common DNA bases in the present reaction would be in the order G > A > T, C. This trend is consistent with the order of the redox potentials of the bases. Such base-selectivity in the reaction, as well as the analyses of the sites and the frequencies of the damage (Table 1), suggests that redox mechanisms are involved in the generation of the DNA damage not only at the position next to the 8-oxo-G, but also at the neighboring positions.

Measurement of the damage of double-stranded DNA

We next studied the permanganate reaction of duplexes that contain 5'- or 3'-end-labeled strands with 8-oxo-G (1, 2, 4, 5 and 7) and their complementary strands, c1, c2, c4, c5 and c7 (Table 3).

Possible mechanisms for neighboring nucleotide damage

To investigate the mechanisms of this reaction, a mixture of a non-labeled strand (2, 7 or 8) and an excess of its control 5'-end-labeled strand was treated with KMnO$_4$ and then with piperidine. The control strand was not cleaved at any of the G positions, and the resulting scissions at the T sites of the control strand were unchanged in their positions and frequencies, as compared with the scissions in the absence of the 8-oxo-G-containing strand. Similarly, the permanganate oxidation of the control strand in the presence of a large excess (300-fold to the oligomer) of 8-oxo-dG did not induce oligomer damage (data not shown). These results suggest that inter-molecular participation (diffusional contact) by the oxidation product(s) of the 8-oxo-G residue is not involved in the induction of the damage. The participation of diffusible oxidants (hydroxyl radicals and singlet oxygens), which might be generated by the oxidation of the 8-oxo-G residue, would be eliminated based on the results that the induction of the DNA damage was not inhibited when the permanganate oxidation of the 8-oxo-G-oligomers 3 and 8 was carried out with a reaction buffer containing a large excess (500-fold to oligomer) of sodium formate (a hydroxyl radical scavenger) or sodium azide and histidine (singlet oxygen quenchers) (data not shown). The superoxide anion is relatively unreactive with DNA (21) (but it can cause DNA damage via the conversion to a hydroxyl radical). Also, the 8-oxo-G-containing DNAs were insensitive to hydrogen peroxide, as described later.

Kinetic studies of DNA damage

The base-selective DNA damage is induced by the oxidation of an 8-oxo-G residue and would be caused by an intra-molecular oxidation. Each nucleoside component was separated under the conditions described in Materials and Methods.

Figure 3. Reversed-phase HPLC chromatogram of the snake phosphodiesterase and alkaline phosphatase digests of single strand 4 before (A) and after (B) the oxidation. Each nucleoside component was separated under the conditions described in Materials and Methods.
Each duplex has an 8-oxo-G-G pair, and the reaction buffer contained 100 mM NaCl to allow the oligonucleotides to form duplexes. The scission at the 8-oxo-G and the neighboring positions was diminished, as compared with the cases of the single strands (Table 3); the cleavage yields of the 8-oxo-G-containing single strands were independent of the absence or presence of NaCl in the reaction buffer. However, it should be noted that whereas the degree of the damage at the T sites of the control duplexes was very low (0–5%), the damage at the T sites in their modified duplexes 5c5 and 7c7 was more frequent. In addition to these observations, a slight strand scission was found to occur at the C position 5′-adjacent to the 8-oxo-G in strand 1 of the duplex, although the scission was not observed at this position on the single strand 1 (Table 1). Furthermore, the reactions of the 5′-end-labeled complementary strands c1, c2, c4, c5 and c7, without 8-oxo-G in the duplexes, were also examined. In contrast to the lack of cleavage of c1, a small amount of strand scission (2.3% yield) was detected at the fifth G position from the 5′-end of the duplex, although the scission was not observed at this position in the single strand 1 (Table 1). These results indicate the existence of uncleavable damage at the 8-oxo-dG site. Similarly, the decrease of dA in duplex 4c4 was examined, each strand had a dA residue. If the reactivity of the 8-oxo-G-containing strand was similar to that of the single-stranded 4, and the dA near the 5′-end of c4 was insensitive to the permanganate treatment, a decrease of ∼35% in the amounts of dA would be expected, because an ∼70% decrease has been observed for the single-stranded 4. The fact that the amounts of dA were less diminished (12% decrease) than in the case of the single strand suggests that the damage of the A residue is suppressed in the duplex form, as in the cases of the other DNA bases.

We oxidized the duplexes containing strands c2A, c2T and c2G, which have A, T and G bases, respectively, opposite the 8-oxo-G contained in the complementary 2 (Table 3), and examined the G damage of the 8-oxo-G (= G)-core (5′-GCC-3′•3′-CNG-5′) in the duplexes. The damage of 2 at the G (5′)-next to the 8-oxo-G, which was in a ‘mismatched base pair (N = A, T, G)’, was higher than that for the parent duplex (2•2) with a ‘matched 8-oxo-G-C base pair (N = C)’. The reactivity of the G site seemed to correlate with the electron donating activity of the N bases; the reactivity increased according to the order G > A > T > C. On the other hand, the damage in the c2 series occurred at the site opposite to the 8-oxo-G and/or at its 5′-adjacent site. Comparison with the strand cleavage efficiency at the G site 5′-adjacent to the N base in the c2 series indicated that the N bases opposite the 8-oxo-G may also affect the reactivity of the G site; the reactivity increased according to the order G, A > T, C.

**Effects of molecular oxygen and other oxidants on the oxidation**

To examine the participation of molecular oxygen in the DNA damage, nitrogen gas was bubbled into the buffer solutions of the single-stranded substrates (3, 4 or 8) prior to the permanganate oxidation. Analysis of the products showed that the cleavage yields at the G, C and T residues in the 5′-end-labeled strands (3 and 8) were unchanged, even after the removal of the O2 (Table 1). Also, the degree of damage of the A residue in 4 was unchanged after the exclusion of O2 (data not shown). Thus, the participation of O2 in the generation of the DNA damage is ruled out.

We also tested oxidation using the oligomers listed in Table 1 with various oxidants that have different oxidizing abilities and either contain heavy metals (A) or not (B); (A) potassium ferricyanate, potassium perchromate, osmium tetroxide, ferric chloride, copper sulfate and cytochrome-c; (B) sodium nitrate, hydrogen peroxide, potassium permanganate, potassium permanganate, potassium permanganate, DDQ (2,3-dichloro-5,6-dicyanobenzoquinone) and oxone (2KHSO5•KHSO5•K2SO4). The reactions were performed under conditions similar to those for the permanganate treatment. However, little cleavage was found with any of these oxidants at the 8-oxo-G and the neighboring residues, after piperidine treatment.

**Effect of continuous base residues on the oxidation**

If the electron transfer reaction participates in the generation and the migration of the damage, and it occurs mainly through π-electrons within the continuous bases of an oligomer, the

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**Table 3. Sequences, cleavage positions and percentages of 5′- or 3′-end-labeled double strands**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>Cleavage yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5′GCCGCACGCGGGT3′</td>
<td>4.9 41(20) (17)</td>
</tr>
<tr>
<td>c1</td>
<td>3′GCCGCACGGCA5′</td>
<td>3.8 30(45)</td>
</tr>
<tr>
<td>2</td>
<td>5′GCCGCACGCGGGT3′</td>
<td>2.7 5.6</td>
</tr>
<tr>
<td>c2</td>
<td>5′GCCGCACGGCA5′</td>
<td>6.4 39(45)</td>
</tr>
<tr>
<td>3</td>
<td>5′GCCGCACGCGGGT3′</td>
<td>3.5 7.7</td>
</tr>
<tr>
<td>c3</td>
<td>3′GCCGCACGGCA5′</td>
<td>0.7 4.5</td>
</tr>
<tr>
<td>4</td>
<td>5′GCCGCACGCGGGT3′</td>
<td>15.2 28</td>
</tr>
<tr>
<td>c4</td>
<td>5′GCCGCACGGCA5′</td>
<td>7.0 7.5</td>
</tr>
<tr>
<td>5</td>
<td>5′GCCGCACGCGGGT3′</td>
<td>7.5 7.5</td>
</tr>
<tr>
<td>c5</td>
<td>3′GCCGCACGGCA5′</td>
<td>8.1 6.4</td>
</tr>
</tbody>
</table>

*All reactions were performed under the conditions described in Materials and Methods. G and bold letters refer to 8-oxo-G and cleavage sites, respectively. Cleavage yields in parentheses were obtained from the experiments with 3′-end-labeled oligomers. Cleavage percentages correspond to those for sites with 5′ to 3′ shown at the left, and the underlined percentages are for 8-oxo-G residues. The percentages for the damage of the T and 8-oxo-G sites are corrected values, as described in Table 1.*
degree and the extent of the damage around the 8-oxo-G may be decreased by the insertion of an abasic region at sites near the 8-oxo-G and the next base. To test the effects of the bases on the DNA damage, we synthesized DNA oligomers with substitutions of the various normal residues of oligomers 2, 7 and 8 with one or more tetrahedrofuran derivatives (Fs), which mimic the structure of an abasic site and we carried out the permanganate oxidation (Table 4). The cleavage at the substituted sites was negligible (data not shown) and it seemed that the disappearance of the damage at these sites was compensated for by the appearance of damage at other sites; that is, there may be whole damage deposition. In the case of strand 10, which has the sequence of strand 10 with the substitution of F for the G 5′-flanking the 8-oxo-G, the oxidation yielded new damage (6.0% yield) at the G site four residues away from the 8-oxo-G. Also, for strand 13, enhancement of the damage (16%) was observed at the G site four residues away from the 8-oxo-G, as compared with the case of the parent 7. For strand 18, which lacks Gs, the damage occurred efficiently at the C site adjacent to the 8-oxo-G, and the cleavage yield at the C was 21% (for the parent 8, 6.6%). While these results indicate that continuous base alignment is not essential for the generation of damage, they do not preclude damage migration through continuous bases.

### Table 4. Sequences, cleavage positions and percentages of 5′- or 3′-end-labeled oligonucleotide derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>Cleavage yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5′GCGCGCgcccGG3′</td>
<td>6.0 (45.5) 12 (8.6) (3.6)</td>
</tr>
<tr>
<td>11</td>
<td>5′GCGPPFPCCGG3′</td>
<td>19 55</td>
</tr>
<tr>
<td>12</td>
<td>5′GCG-Hex-gcccGG3′</td>
<td>17 29</td>
</tr>
<tr>
<td>13</td>
<td>5′ATGATATATA3′</td>
<td>32 23</td>
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<td>14</td>
<td>5′ATGATATATA3′</td>
<td>18 31 13</td>
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<td>30 32 27</td>
</tr>
<tr>
<td>18</td>
<td>5′TTTTTTTTTT3′</td>
<td>28 21 41</td>
</tr>
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<td>19</td>
<td>5′ATG-Hex-gATATA3′</td>
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<td>5′ATG-(Hex)-gATATA3′</td>
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<td>21</td>
<td>5′ATG-(Hex)-gATATA3′</td>
<td>28 19</td>
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</tbody>
</table>

*All reactions were performed under the conditions described in Materials and Methods. G and bold letters refer to 8-oxo-G and cleavage sites, respectively. Cleavage yields in parentheses were obtained from the experiment with the 5′-end-labeled oligomer. Cleavage percentages correspond to those for sites with 5′ to 3′ shown at the left, and the underlined percentages are for 8-oxo-G residues. The percentages for the damage of the T and 8-oxo-G sites are corrected values, as described in Table 1.*

Further studies using 11, 15 and 16 (5′-end-labeled) showed that as the numbers of F-substitutions increase in the region between the G site to be damaged and the 8-oxo-G, the G site becomes damaged more frequently, as shown in Table 4. These results suggest that the bases intervening between the 8-oxo-G and the G suppress the damage of the G. On the other hand, the reaction of the 3′-end-labeled 10 showed an increase in the respective yields of the cleavages at the damaged sites on the 3′-side of the 8-oxo-G, as compared with the case of the parent 2. Since the results of the oxidations with 4, 5 and their parent 2, which contain A, T and G, respectively, 5′-next to the 8-oxo-G can also be explained in terms of whole damage deposition, the results obtained from the F-substituted oligomers would not be due to structural changes of the substrates caused by the introduction of the F residue(s).

**Effect of poly (alkylether) linkers instead of the sugar–phosphate backbone on the oxidation**

We next examined the possibility of the transfer of damage (e.g. radical cation species, see Discussion) via a sugar–phosphate backbone (24,25). If the sugar–phosphate backbone is an essential medium for the damage migration, the replacement of F by a linker lacking a sugar–phosphate skeleton in the oligomer may diminish the damage of the nucleotide (dG) joined with the 8-oxo-G residue via the linker. Thus, we carried out the synthesis and the permanganate oxidation of oligomers, in which two oligomer fragments were linked with a hexa (ethylene glycol) unit (Hex) via two phosphodiester (12 and 19 in Table 4). Since the linker is three nucleotides long (∼20 Å), the results of the strand cleavage analysis were compared with those of strands 11 and 16 with three continuous F residues. The results shown in Table 4 indicated that the cleavage yields at the 8-oxo-G and the G sites were almost equal for 12 and 11 and for 19 and 16. All of our results argue against a direct role for the sugar–phosphate backbone in damage migration.

In order to test whether the damage migration occurs in DNA oligomers linked by a long linker, we prepared substrates 20 and 21 containing a long spacer composed of two and three continuous Hex residues, respectively. These linkers, (Hex), and (Hex), are 45 and 70 Å long, respectively. The oxidation of the 5′-end-labeled substrates revealed that the damage at the G site joined with the 8-oxo-G via the linker was significant. Also, from the results, it appears that as the length of the linker increases, the damage of the G becomes less efficient (Table 4); the cleavage yields at the G site of the substrates (19, 20 and 21) with (Hex), (Hex), and (Hex) were 56, 45 and 28%, respectively.

### Inhibition of the DNA damage by nucleosides: intra-molecular reaction versus inter-molecular reaction

We propose that the generation of the neighboring base damage involves an intra-molecular redox process. If the electron transfer process is truly involved in the generation of the DNA damage, then the G damage in the modified DNAAs may be decreased by the addition of deoxynucleosides, and the efficiency of the inhibition would be in the order dG > dA > dC. These nucleosides are inert to permanganate oxidation, whereasdT is not, under the present conditions.

Thus, we examined the effects of the additives (dG, dA and dC) on the generation of the damage at the G sites of strands 19–21. The permanganate oxidation of the oligomers was carried out in the presence of various concentrations of the nucleosides and then the strand cleavage analysis was performed. Experiments with strand 19 containing a Hex linker indicated that none of the nucleosides (−150-fold excess to the G residue) inhibited the generation of the damage at the G site (data not shown); that is, the intra-molecular reaction was not inhibited by the additives and the inter-molecular (between DNA molecules) reaction is unlikely. As
experiments (26). The mechanism of the inhibition probably
involves inhibition, by dG and dA, of the intra-molecular redox
reaction in the DNA oligomer containing the spacer; inter-mol-
cular electron transfer between the nucleoside and the DNA
leads to the inhibition. The results also demonstrate that the
inhibition of dG becomes effective as the distance (spacer)
between the 8-oxo-G and the G residue is longer. In addition,
using oligomer 21, we found that sodium formate, sodium azide
and histidine did not inhibit the generation of the DNA damage.

**DISCUSSION**

**Mechanisms including redox chemistry in base damage**

From the results of various experiments for single-stranded DNA,
we found that the reactivity of the common bases in the
permanganate oxidation of DNA oligomers with a single
8-oxo-G residue is in the order G > A > T, C (Tables 1 and 2).
The tendency is consistent with the order of the electron donating
activity of the bases (22,23). This was also found for the inhibitory
effect of the common deoxynucleosides (dG > dA > dC) in the
oxidation of the oligomer containing a long non-nucleotide
spacer (Fig. 4). These results suggest that a redox mechanism
participates in the generation of the damage of single-stranded
DNA.

In double-stranded DNA with the 8-oxo-G in one strand,
inter-strand base damage as well as intra-strand base damage was
observed at the bases close to 8-oxo-G, but with lesser efficiency
than that of the single strand (Table 3). The pathway involving
redox chemistry for the double strand damage was suggested by
the strand cleavage analysis of the damage to the 8-oxo-G-core
(5’-GGC-3’ 3’-CNG-5’) in 2×42 and the analogs. The damage at
the two G sites in the core was influenced, according to the N
base residue opposite to the 8-oxo-G, and the damage of the Gs
increased in the order G ≥ A > T ≥ C. The base selectivity for
the damage is similar to that for the single strand.

From the above observation and results that reactive oxygen
species and dioxygen may not participate in the neighboring base
damage, we speculate a mechanism for the damage induction. It
is well known that ionizing radiation (25,27) and photoirradiation
with photosensitizers (28–32) to DNA cause oxidative damage,
mainly at guanine sites. In the reactions, radical cations (often
called positive holes) of the bases are formed, and they intra-
molecularly migrate within single-stranded DNA (33–35) and
double-stranded DNA (33,36,37) according to the electron
donating activities of the bases. Namely, the holes of T*, C*, and
A* tend to transfer to G to produce G**. If such a reaction has
occurred in the present cases, a reactive species (oxidant) might
be a transient intermediate generated from the KMnO4 oxidation
of the 8-oxo-G residue, and the resulting residue might oxidize
the neighboring inter- and intra-strand bases to afford the radical
cations, which would react with H2O and/or KMnO4. Recently, we
carried out the KMnO4 reaction using 5’-O-tert-butyldimethyl-
silyl-7,8-dihydro-8-oxo-2’-deoxyguanosine as a model. On the
basis of the characterization of isolated three main products, each
of which had an intact sugar residue, the products were
considered to be produced via oxidation of the purine 4,5-double
bond (the results will be published by M.Fukuoka et al. (1998)
Nucleic Acids Symp. Ser. No.39). Similar reactivity of the double
bond has been observed in the reaction of an 8-oxo-guanosine
derivative with singlet oxygen (38). We envisioned the structure
(or its deprotonated form) depicted in Figure 5 as the intermediate
described above, which might be produced by the double bond
oxidation followed by elimination of water molecule(s) and
expected to have oxidizing ability. Its analogous structure has
been proposed as an electrochemical oxidation intermediate of
9-methyluric acid (39). Although the reason for the apparent low
reactivity of 8-oxo-G-containing duplexes to the neighboring base
damage as well as mechanisms of the damage formation is
unclear, we could imagine the inaccessibility of attacking agents
(KMnO4 or H2O) to the bases (or holes), owing to the more
anionic and more rigid character of double-stranded DNA.

Mechanistic proposals to explain the observed redox chemistry
can be divided into two general classes: electron transfer
mediated by stacked bases and an intramolecular reaction with
the activated 8-oxo-G intermediate by direct contact. The former
mechanism has been proposed in DNA-mediated photochemistry
(36,37) and in oxidative DNA damage by ionizing radiation (34).
The relative importance of the two damage migration pathways is likely to be substrate-dependent. For example, the observation of damage at a G site four bases away from the 8-oxo-G in the double strand with 7 (Table 3) is most easily explained by electron transfer through stacked bases since the double helix would prevent direct contact between these two bases. By contrast, a direct contact mechanism offers a more plausible explanation for the damage migration observed for 8-oxo-G oligomers containing abasic spacers.

Mechanism of strand cleavage

Since the ladders observed by the strand cleavage analysis are comparable to Maxam–Gilbert sequencing ladders, the induced strand scissions may be due to the formation of an abasic site as an intermediate. However, DNA cleavage did not occur upon heating without piperidine (data not shown). Therefore, a ‘regular’ abasic site and a furanone site, which is also sensitive to heating without piperidine, would not be considered as proper candidates for those remnants. It is also known that oxidation-induced sugar damage affords sites leading to frank strand scission or heat-labile sites (21): the remnants should contain degraded bases. A ureido sugar is a plausible candidate. It is known to be produced by permanganate oxidation of DNA from T, C and G residues under certain conditions, and this damage is labile to alkaline treatment and causes strand breakage (19). Also, 8-oxo-G can be considered as a precursor of a damaged G remnant, since 8-oxo-G can be generated by the reaction of G** with H2O (15,28,29). However, it would not be a direct precursor for strand scission at the G sites, because 8-oxo-G in DNA yields only slight strand scission at the site upon piperidine treatment, and it is definitely permanganate-sensitive.

In a biological sense, our results about neighboring base damage have important implications. If such DNA damage involving 8-oxo-G occurs in cells, it would cause serious lesions in living organisms. Therefore, it is worthwhile to examine whether the damage migration can occur under conditions closer to those that might be observed within cells subjected to oxidative stress. This project and studies to confirm the redox process and to characterize the damaged base residues are in progress in our laboratory.

MATERIALS AND METHODS

Materials

Nucleosides dG, dA and dC were from Yuki Gosei Kogyo Co. 8-oxo-dG was prepared according to the published method (13) with a slight modification. The common phosphoramidites for DNA synthesis were obtained from Applied Biosystems, and tetrahydrofuran- and hexa(ethylene glycol)-phosphoramidites were from Glen Research. Oligonucleotides with or without 8-oxo-G were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer using the standard solid-phase cyanoethyl phosphoramidite method, deblocked and purified as described (13,40). All solutions used in the study were made with water sterilized after prior purification by a Millipore Milli-Q water purification system.

Analysis of strand cleavage

The single-stranded oligonucleotides were labeled at the 5′- or 3′-end with T4 polynucleotide kinase (Takara) plus [γ-32P]ATP (Amersham) or with terminal deoxynucleotidyl transferase (Takara) plus [α-32P]dATP (Amersham), respectively. Solutions (20 µl) buffered to pH 7.0 (10 mM sodium phosphate) and containing 0.5 µM oligonucleotide (a mixture of 10 pmol non-labeled strand and 0.1 pmol labeled strand) and 120 µM permanganate, were maintained at 25°C for 15 min and were quenched with 5 µl of allyl alcohol. Quenching of the reaction with the other oxidants, except for cytochrome-c, was performed by ethanol precipitation of the oligomers from 0.3 M sodium acetate solution containing 0.1 mM EDTA and 0.0025% rRNA. The cytochrome-c was removed by extraction with phenol/chloroform/isoamyl alcohol. Exclusion of molecular oxygen was carried out by bubbling N2 into the reaction buffer and the permanganate solution. For duplex formation, the labeled single strands were annealed with the complementary strands by heating the buffered DNA solution (pH 7.0, 10 mM sodium phosphate, 100 mM NaCl) at 80°C for 5 min. This DNA solution was slowly cooled to room temperature and was used for the permanganate reaction; in the case of the single strands, all KMnO4 reactions were performed in buffer lacking salts such as NaCl, in order to avoid the formation of higher ordered structures, such as a G tetraplex DNA (41). The samples (26 µl) were individually added to 25 µl of 2 M piperidine, heated at 90°C for 30 min, lyophilized to dryness, coevaporated with water (40 µl × 3) and dissolved in gel loading solution containing 5 M urea, 0.1% xylene cyanol and 0.1% bromophenol blue. The samples were heated to 55°C and chilled quickly for 20% polyacrylamide gel (denaturing 7 M urea) electrophoresis. The radioactivities of the fractionated, cleaved products on the gel were analyzed with a Bioimaging analyzer (Fujix BAS 2000). The cleavage yield (%) was obtained from the calculation: [radioactivity of each band/total radioactivity of the bands including the band of the remaining substrate] × 100.

Analysis of the nucleoside composition of the oxidized DNA

The single-stranded oligonucleotides were dissolved in 2 ml of 10 mM sodium phosphate buffer (pH 7.0) to a 0.5 µM concentration. The double-stranded solutions were prepared similarly, with 10 mM sodium phosphate buffer containing 100 mM NaCl. The permanganate reaction was carried out at a KMnO4 concentration of 120 µM at 25°C for 15 min. The oxidized oligomers were desalted by gel filtration on Sephadex G-25 (NAP-5, Pharmacia) and were then digested with snake venom phosphodiesterase and alkaline phosphatase according to the reported method (40). The resultant nucleoside mixture was analyzed by reversed-phase HPLC on an Inertsil ODS 2 column (4.6 × 250 mm) and was detected by UV absorption at 254 nm; elution was performed with a linear gradient of acetonitrile (from 0 to 7.5% in 50 min) in 0.1 M triethylammonium acetate, at a flow rate of 0.5 ml/min. The decreases in the percentages of the nucleosides were calculated based on a comparison of the ratio of (peak areas on the HPLC profile/molar absorption coefficients) of each nucleoside before and after the oxidation of the oligonucleotides.
Reaction kinetics

The oxidation was performed with 5′-end-labeled single strands (0.5 μM) and KMnO$_4$ (120 μM) within 5 min of the reaction initiation under the conditions described above, and in the strand cleavage analysis, the remaining substrates (r.s.) were quantified by radiodensitometry as a function of time (t). The numbers of piperoxane-induced 8-oxo-G scissions were subtracted from those induced by 8-oxo-G oxidation. The regression of the resulting net remaining substrates as a function of time was fit to a pseudo-first-order condition and rate constants were obtained from the slope of ln (r.s.) versus t plot.

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