Tandem Base Lesions Are Generated by Hydroxyl Radical within Isolated DNA in Aerated Aqueous Solution

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Abstract: The hydroxyl radical-mediated formation of two tandem base lesions within DNA including *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)formylamine-8-oxo-7,8-dihydro-2′-deoxyguanosine (dβF-8-oxodGuo) and 8-oxodGuo-dβF is reported in this study. A specific enzymatic processing was developed to quantitatively release the tandem lesions as dinucleoside monophosphates from DNA. Then, the resulting hydrolyzed DNA samples were analyzed using liquid chromatography coupled to electrospray ionization tandem mass spectrometry. The simultaneous measurement of the two vicinal lesions was performed in the negative mode using the accurate and sensitive multiple reaction monitoring technique. For this purpose, two characteristic ions arising from the fragmentation of the pseudo-molecular ion $[M - H]^-$ were monitored. Both dβF-8-oxodGuo and 8-oxodGuo-dβF damage were found to be generated in γ-irradiated DNA as a significant fraction of •OH radical-induced base damage. Interestingly, 8-oxodGuo-dβF was produced in a much higher yield than the reversed sequence lesion. Indirect evidence is provided for the formation of other tandem lesions involving 8-oxodGuo, but that still remain to be fully identified. Insights into the mechanism of formation of the DNA damage were gained from several experiments including DNA photosensitization, γ-irradiation in the presence of iron, and exposure to Fenton reagents. This allowed refinement of the proposed pathways for the formation of dβF-8-oxodGuo and 8-oxodGuo-dβF tandem base damage.

Introduction

A great deal of information is available on the main classes of •OH radical-mediated DNA lesions including altered bases, strand-breaks, abasic sites, and DNA−protein cross-links.[1−3] During the last three decades, efforts were made to isolate and characterize oxidative damage to purine and pyrimidine bases from model compounds and isolated DNA.[3,4] Recently, unexpected types of damage involving two adjacent bases have been shown to be generated in short oligonucleotides upon exposure to X-rays in aqueous solution.[5] It was proposed that these tandem lesions resulted from a single free radical initiating event. Most X-ray-induced tandem base lesions involve guanine and an adjacent pyrimidine base. These lesions include *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)formylamine-8-oxo-7,8-dihydro-2′-deoxyguanosine (dβF-8-oxodGuo) and its isomer 8-oxodGuo-dβF in aerated solution. Several compounds arising from the addition of thymine to the guanine moiety under anoxic conditions also have been isolated.[6−9] More recently, a thymine−guanine adduct has been found to be generated upon type I photosensitization in aerated aqueous solution.[10] Moreover, it was postulated that tandem base damage may include modified bases not immediately adjacent to each other.[7] In all cases, tandem lesions were produced in significant yields. Double lesions are suspected to exhibit deleterious biological effects.[11] Indeed, the repairability of such damage is strongly influenced by the location and the nature of the lesions on either the same or the opposite strand.[12−16] Inhibition of base excision repair enzymes has been observed together with the formation of repairable single strand-breaks and potentially lethal double strand-breaks. Preliminary works have shown that the dβF/8-oxodGuo lesions were substrates for both *E. coli* Fapy-DNA-N-glycosylase (Fpg) and endonuclease III repair enzymes.[17] However, the tandem lesions are not completely excised by the latter repair enzymes.

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Emphasis was placed in the present work on the measurement of dβF-8-oxodGuo and 8-oxodGuo-dβF dinucleoside monophosphates within isolated DNA upon exposure to γ-rays in aerated aqueous solutions. It should be remembered that under the latter conditions, •OH radicals arising from the radiolysis of water molecules are the predominant reactive species. Until now, no assay was available for the measurement of the tandem lesions partly because of the lack of a quantitative hydrolysis procedure. In a recent study, we investigated the activity of several nucleases toward dβF/8-oxodGuo inserted within synthetic modified oligodeoxynucleotides.[17] A reliable enzymatic hydrolysis assay involving the simultaneous use of 3′- and 5′-exonucleases was found to quantitatively excise the tandem lesions as dinucleoside monophosphates from modified oligodeoxynucleotides. A similar approach was applied in the present study to DNA oxidized by •OH radicals in aerated aqueous solution. Interestingly, the two isomeric tandem lesions were efficiently separated by high performance liquid chromatography on an octadecylsilyl silica gel (ODS) column. Detection of the modified dinucleoside monophosphates was achieved at the output of the column using the highly accurate electrospray ionization tandem mass spectrometry (ESI-MS/MS) technique. Tandem lesions appear to be important components of •OH-mediated oxidative damage to DNA. Additional experiments in which DNA was exposed to photosensitizers, γ-rays in the presence of iron, or a Fenton reagent provided relevant mechanistic information.

Results and Discussion

Isolation and Characterization of Tandem Base Damage Standards. Vicinal base lesions, namely dβF-8-oxodGuo, dαF-8-oxodGuo, 8-oxodGuo-dβF, and 8-oxodGuo-dαF, were obtained from either 4-mer or 25-mer synthetic oligonucleotides, including d(AFβG°C), d(AFαG°C), d(AG°FβC), d(AG°FαC), d(CAGTACGTAGGAGFβG°CCATCGATAG), and d(CAGTACGTAGGAG°FβGCCATCGATAG). Enzymatic processing by snake venom phosphodiesterase (VPD) and calf spleen phosphodiesterase (SPD) (3′- and 5′-exonucleases, respectively) has already been optimized in a previous study.[17] The hydrolysis along the latter modified DNA fragments by either 3′-exonuclease or 5′-exonucleases has been followed by MALDI-TOF mass spectrometry.[18] Both phosphodiesterases were found to be unable to bypass the dβF-8-oxodGuo and 8-oxodGuo-dβF lesions. As a consequence, the latter tandem lesions are completely resistant toward digestion by the two phosphodiesterases. Thus, the dinucleoside monophosphates were released from modified oligodeoxynucleotides by sequential digestion with VPD and SPD. The resulting dinucleoside monophosphates were separated by high performance liquid chromatography and characterized by ESI-MS/MS (vide infra) and UV spectroscopy. All features were identical with those of authentic compounds.

DNA Hydrolysis. Reliable measurement of modified bases in isolated DNA requires a quantitative release of the studied lesions. In addition, the targeted compound has to be stable under the hydrolysis conditions. As previously described, experiments using oligodeoxynucleotides that contained tandem base lesions showed that the combination of nuclease P1 and alkaline phosphatase for the hydrolysis was not appropriate to isolate dβF-8-oxodGuo and 8-oxodGuo-dβF.[18] Indeed, dβF-8-oxodGuo was found to be partly hydrolyzed, whereas 8-oxodGuo-dβF was completely digested into nucleosides. In contrast, oligodeoxynucleotide digestion by snake venom and calf spleen phosphodiesterases was blocked at the site of the tandem base damage as shown by MALDI-TOF MS analysis. The latter hydrolysis protocol was extended to isolated DNA with slight modifications. First, the micrococcal nuclease (MN) digestion converted the isolated DNA into smaller fragments carrying a phosphate group at the 3′-end and a 5′-OH terminus. The sizes of the resulting DNA fragments were assessed by agarose gel electrophoresis. They were found to be smaller than 200 nucleotides. Then, the sequential use of exonucleases allowed the quantitative release of the tandem base lesions. After the digestion from the 5′-end to the 3′-end by SPD, an hydrolysis step involving alkaline phosphatase was performed to remove the 3′-phosphate groups. In the final step, the action of VPD (3′-OH to 5′-OH) allowed the complete release of the vicinal base damage as dinucleoside monophosphates.

Several parameters were investigated to establish the completion of the enzymatic digestion. First, the enzymatic hydrolysis was carried out either by adding various amounts of micrococcal nuclease or by increasing times of incubation for either SPD or VPD. The amount of tandem lesion released first increases and then reaches a maximum. No variations in the amount of tandem lesions detected within irradiated DNA were noticed for higher enzyme concentration or longer incubation times. Therefore, hydrolysis was carried out with 0.75 unit of micrococcal nuclease, 0.012 unit of SPD, and 0.0018 unit of VPD. The respective incubation times were 30 min, 3 h, and 15 min. Analysis of the sample by HPLC with UV detection revealed that the whole DNA was digested into nucleosides since the sums of detected nucleosides correspond to the amount of injected DNA.

HPLC-ESI-MS/MS Measurement of Tandem Base Damage. Our goal was to develop a quantitative method for the simultaneous measurement of dβF-8-oxodGuo and 8-oxodGuo-dβF tandem base damage within DNA exposed to γ-radiation in aerated aqueous solution. Electrospray ionization in the negative mode combined with multiple reaction monitoring (MRM) provides a highly specific and sensitive detection. Under the latter ionization conditions, the phosphate group is easily ionized and the response of the detector is stable along a series of injections. In addition, HPLC conditions were found to ensure a good separation of the lesions on octadecylsilyl silica gel (ODS) column (capacity factors $k' = 1.95$, 2.2, 2.35, and 3.4 for dβF-8-oxodGuo, dαF-8-oxodGuo, 8-oxodGuo-dαF, and 8-oxodGuo-dβF, respectively). Full scan mass spectra of the lesions in the negative mode exhibited a pseudo-molecular ion $[M - H]^-$ at m/z 505. Two main fragments were observed in the MS/MS spectra, at m/z 166 and 362, corresponding to [8-oxoGua (8-oxo-7,8-dihydroguanine)-H]$^-$ and the [(8-oxo-7,8-dihydro-2′-deoxyguanosine phosphate)-H]$^-$, respectively. However, the relative yield of the daughter ions was dependent on the tandem lesion observed. The ion with a mass of 362 was the major ion detected upon fragmentation of dβF-8-oxodGuo, whereas its abundance was very low in the mass spectrum of 8-oxodGuo-dβF (Figure 1). As a consequence, the multiple reaction monitoring (MRM) mode was applied to simultaneously monitor the two major transitions 505 → 166 and 505 → 362. The relative intensity of the latter signals allowed the identification of the peaks observed in the chromatograms to be confirmed. Interestingly, the sensitivity of the assay was found to be high with a limit of detection close to 10 fmol. Moreover, identical responses were obtained when the

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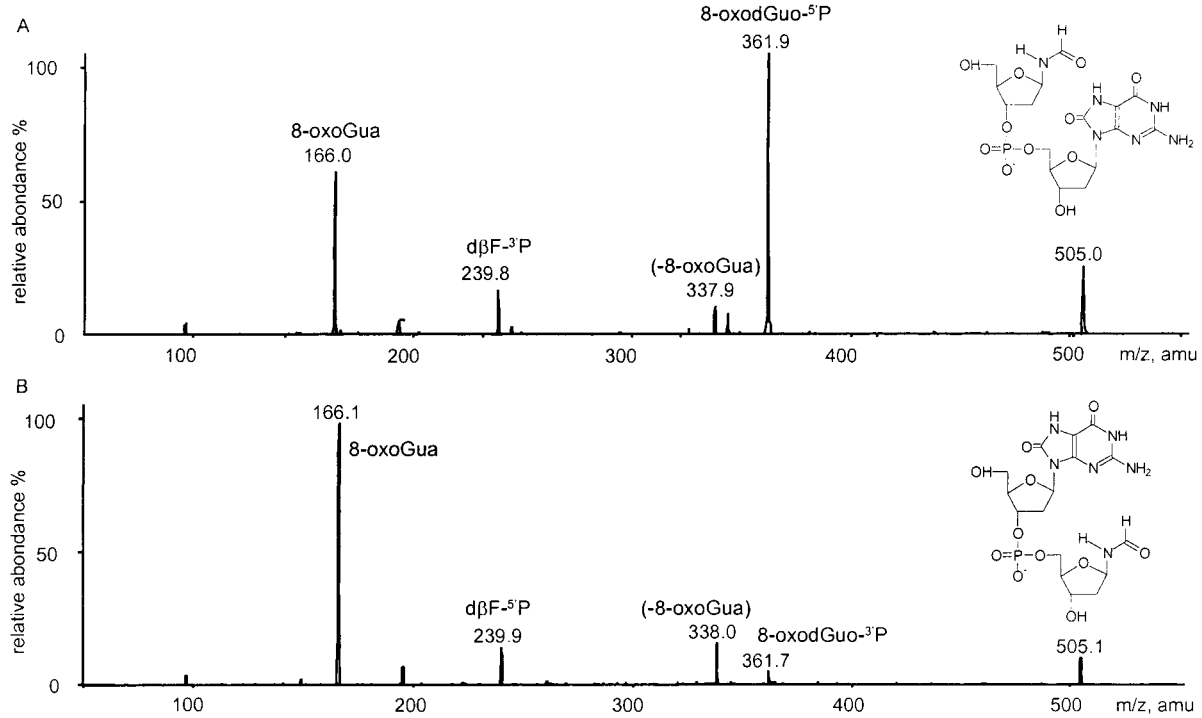

Figure 1. ESI-MS/MS spectrum of dβF-8-oxodGuo (A) and 8-oxodGuo-dβF (B) recorded in the negative mode.

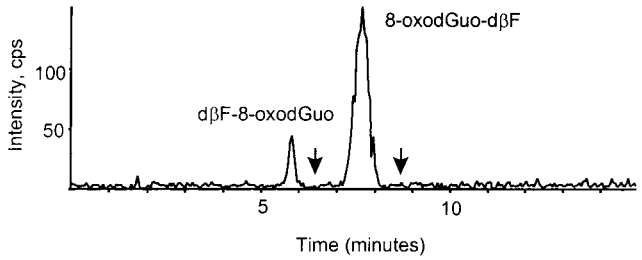

Figure 2. Chromatogram of the analysis of γ-irradiated DNA (10 Gy). Transitions 505 → 166 and 505 → 362 were simultaneously monitored. Arrows represent the expected retention times of the dαF-8-oxodGuo and 8-oxodGuo-dαF anomers.

same amount of authentic lesion was analyzed as a pure sample or mixed with hydrolyzed DNA. This avoids the use of internal calibration which is needed with nucleosides.[19] An external calibration was applied for all quantitative measurement of both tandem lesions.

It has been previously shown that the C-1′ of dβF may undergo anomerization under slightly alkaline or acidic conditions.[17] Therefore, it was determined whether dαF-8-oxodGuo and 8-oxodGuo-dαF are present in the enzymatic DNA hydrolysate. No α anomer product was detected within hydrolyzed DNA (Figure 2).

Measurement of the Tandem Base Damage within Isolated DNA Exposed to γ-Radiation. The results reported here involved several experiments carried out with two different batches of calf thymus DNA. Doses of γ-radiation ranging from 0 to 100 Gy were applied and the measurements were performed as described above. The HPLC-ESI-MS/MS analysis clearly showed that dβF-8-oxodGuo and 8-oxodGuo-dβF were produced within DNA in aerated aqueous solution (Figure 3). The formation of the two tandem base lesions was linear as a function of the dose down to 5 Gy. This value which corresponds to a irradiation time of 15 s represented the lowest dose for which irradiation experiments can be carried out properly with the presently available dose rate. Interestingly, 8-oxodGuo-dβF (radiolytic yield $G = 0.0013$ μmol/J) was produced in a 15-fold higher yield than dβF-8-oxodGuo ($G = 0.0001$ μmol/J).[20] In addition, HPLC-EC measurement of 8-oxodGuo as a single lesion (vide infra) in the same sample revealed that the amount of 8-oxodGuo-dβF was only 10-fold lower that the level of single 8-oxo dGuo ($G = 0.0130$ μmol/J) (Figure 3).

As far as the mechanisms of formation are concerned, the present results confirmed that the tandem lesions are not generated by two independent free radical-initiating events.[21] Even though this mechanism could have been postulated for high doses, 8-oxodGuo-dβF and dβF-8-oxodGuo are generated linearly in significant yields even at low doses (5 Gy). This suggests that the formation of the tandem lesions involves a single radical event as was previously postulated.[5,9]

HPLC-EC Measurement of 8-oxodGuo. The level of total 8-oxodGuo present in irradiated DNA was assessed by HPLC-EC. It was assumed that 8-oxodGuo was quantitatively released upon nuclease P1 and alkaline phosphatase treatment, according to the observations made using 8-oxodGuo-dβF-containing oligodeoxynucleotides and previous measurements.[22] The amount of dβF-8-oxodGuo that could be released as dinucleoside monophosphate upon nuclease P1 treatment was neglected because the yield of the latter lesion was 100-fold lower than that of 8-oxodGuo.

Interesting information was inferred from the comparison of the overall yields of exonuclease-mediated release of 8-oxodGuo-containing products, namely 8-oxodGuo-dβF, dβF-8-oxodGuo, and single 8-oxodGuo with respect to the total level

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(20) A similar linear relationship was observed for the formation of the two tandem base lesions upon exposure of H_2O_2 solution to UV-C radiation. This rules out the implication of the cluster •OH radical in the radiation-induced formation of the tandem lesions.

(21) A similar trend was observed when exposing TpdG and dGpT to ionizing radiation in aerated aqueous solution.

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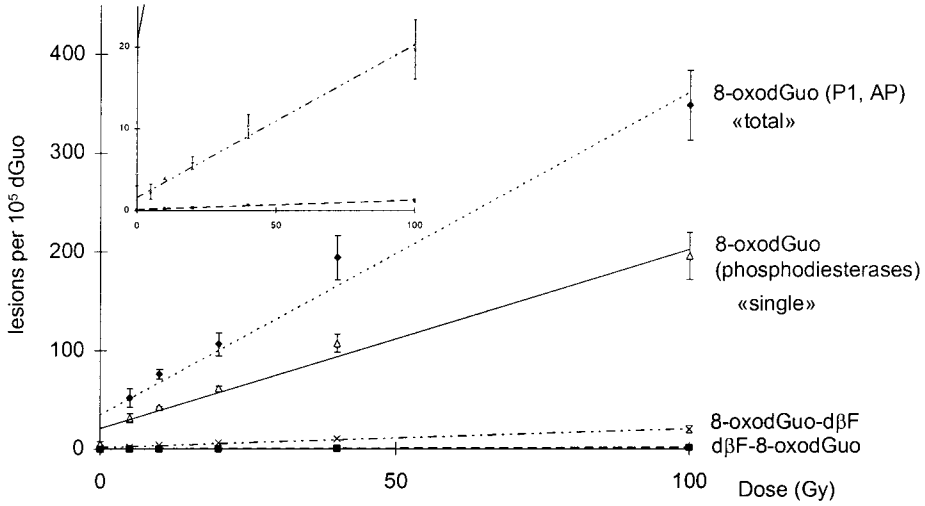

Figure 3. Formation of tandem lesions, single 8-oxodGuo damage, and total 8-oxodGuo modifications within γ-irradiated DNA. The two latter values were determined following phosphodiesterase and digestion and nuclease P1 digestion plus alkaline phosphatase digestion, respectively.

of 8-oxodGuo determined following treatment with nuclease P1 and alkaline phosphatase. As a major result, it appeared that the combined yield of the three former lesions represented only 70% of 8-oxodGuo-containing products following phosphodiesterase hydrolysis. This observation suggests the presence in significant amounts of other nondigested tandem lesions consisting of 8-oxodGuo on one hand and not yet identified damaged bases on the other hand.

The similar level of 8-oxodGuo detected in photosensitized DNA using either nuclease P1 plus alkaline phosphatase or the mixture of 3′- and 5′-exonucleases rules out an incomplete digestion by the applied enzymatic treatment. Indeed, photosensitization reactions were carried out in the presence of either riboflavin or rose bengal. The photoexcited sensitizers were not able to induce any detectable amount of 8-oxodGuo/dβF tandem lesion (vide infra). Measurements of 8-oxodGuo were carried out in samples hydrolyzed using either nuclease P1 together with alkaline phosphatase or the mixture of phosphodiesterases. Interestingly, the level of 8-oxodGuo measured was identical irrespective of the enzymatic system used (Figure 4).

Insights in the Mechanism of Formation. Comparison of the yields of formation of modified bases generated upon either γ-irradiation in the presence of Fe(II)/EDTA, photosensitization or oxidation by Fenton reaction was carried out to gain further insights into the mechanism of formation of dβF-8-oxodGuo and 8-oxodGuo-dβF tandem lesions. Moreover, the measurement of thymidine degradation products including 5-formyl-2′-deoxyuridine (5-FordUrd) and 5-(hydroxymethyl)-2′-deoxyuridine (5-HMdUrd) provided relevant mechanistic information.

Aerated aqueous solution of calf thymus was incubated in the presence of ferrous ion/EDTA complex together with increasing amounts of H_2O_2. In a subsequent step, oxidized DNA was hydrolyzed using either the nuclease P1 and alkaline phosphatase technique or the phosphodiesterase digestion. Both dβF-8-oxodGuo and 8-oxodGuo-dβF were generated under these conditions and the level of formation of the tandem lesions was found to be proportional to the amount of H_2O_2. The yield of formation of 8-oxodGuo-dβF was 30-fold higher than that of dβF-8-oxodGuo but only 5-fold lower than that of the exonuclease-mediated released "single 8-oxodGuo" (Figure 5). In addition, the sum of tandem lesions and single 8-oxodGuo was lower than the overall level of 8-oxodGuo detected using the nuclease P1 and alkaline phosphatase hydrolysis, as observed upon γ-irradiation. This observation strongly suggested again

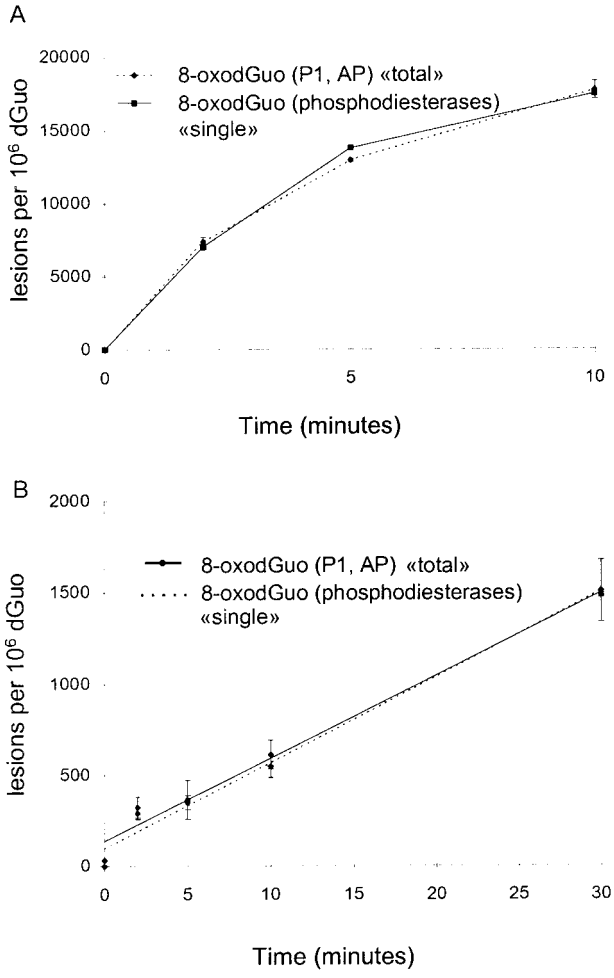

Figure 4. Formation of 8-oxodGuo in riboflavin (A) or rose bengal (B) photosensitized aqueous aerated solution of calf thymus DNA either hydrolyzed using nuclease P1 and alkaline phosphatase or digested with phosphodiesterases.

that the dβF and 8-oxodGuo tandem modifications are not the only ones containing 8-oxodGuo multiple lesions. The relative amount of tandem base lesions generated with respect to single 8-oxodGuo was higher upon Fenton reaction than γ-irradiation. This observation suggested an effect of the presence of the Fe(II) reducing agent.

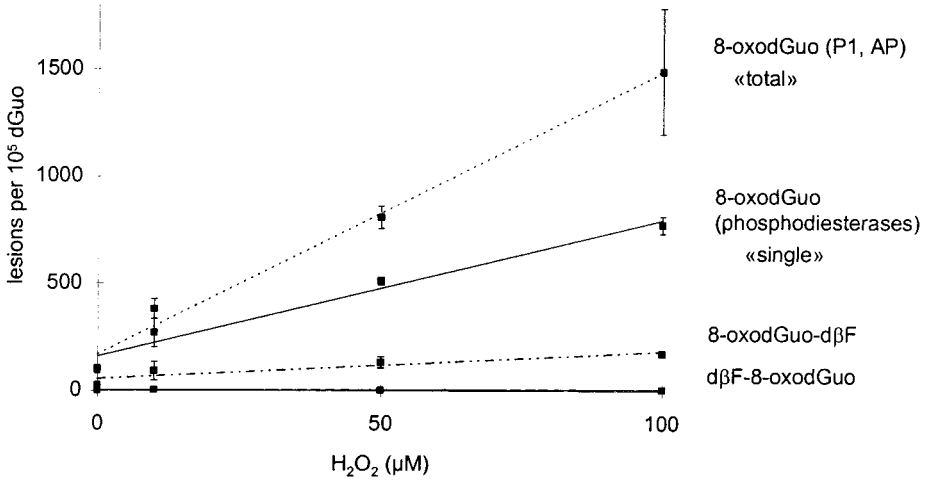

Figure 5. Formation of tandem lesions, single 8-oxodGuo damage, and total 8-oxodGuo modifications in the presence of 10 μM $[Fe^{II}\text{-EDTA}]^{2-}$ and increasing amounts of H_2O_2 within aqueous aerated calf thymus DNA solution.

Table 1. Radiolytic Yield of Formation (μmol·J^{-1}) of 8-oxodGuo-dβF (A) and dβF-8-oxodGuo (B) Lesions upon DNA γ-Irradiation of DNA in the Presence of Increasing Amounts of $[Fe^{II}\text{-EDTA}]^{2-}$

	8-oxodGuo "total"	8-oxodGuo-dβF	dβF-8-oxodGuo
0 μM Fe	2.34×10^{-2}	0.13×10^{-2}	0.01×10^{-2}
10 μM Fe	4.05×10^{-2}	0.31×10^{-2}	0.02×10^{-2}
100 μM Fe	2.52×10^{-2}	0.47×10^{-2}	0.04×10^{-2}

To better assess the role of ferrous ions, γ-irradiation of an aqueous solution of DNA was carried out in the presence of Fe(II)/EDTA. The ferrous iron, as a reducing agent, is able to modify the distribution of the decomposition products by reacting with transient species radicals. The formation efficiency of both vicinal lesions dβF-8-oxodGuo and 8-oxodGuo-dβF increased with addition of ferrous iron (Table 1). In addition, 8-oxodGuo-dβF was generated in a 15-fold higher yield than the rate of dβF-8-oxodGuo. The ratio is similar to those obtained in γ-irradiated DNA in the absence of iron. The amount of 8-oxodGuo generated was also higher when the γ-irradiation was carried out in the presence of 10 μM Fe(II)/EDTA. In contrast, the yield of 8-oxodGuo produced by γ-irradiation was lower in the presence of 100 μM Fe(II)/EDTA than by adding 10 μM Fe^{2+}. Water radiolysis yields hydroxyl radicals and aqueous electrons. The latter species react with molecular oxygen to give rise to superoxide radicals. Then, in the presence of Fe(II) reducing agent, the superoxide radical could be transiently converted into H_2O_2 and subsequently into hydroxyl radicals via a Fenton reaction. As a consequence of the increase in the yield of formation of $^{\bullet}$OH, the yield of 8-hydroxy-7,8-dihydroguan-7-yl reducing radical is expected to increase upon γ-irradiation in the presence of Fe(II)/EDTA. This increase is limited by the amount of available solvated electron produced by water radiolysis. In addition, the presence of Fe(II)/EDTA could affect the conversion of 8-hydroxy-7,8-dihydroguan-7-yl reducing radical. Oxidation of the latter radical would give rise to 8-oxodGuo while reduction would yield 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua).[23,24] Addition of iron will promote the reduction pathway. The two possibilities should be taken into account to explain the formation of 8-oxodGuo. First, addition of a relatively small amount of iron (10 μM) is expected to double the amount of hydroxyl radical through the conversion of aqueous electron. As a consequence, the level of guanine reducing radicals increases. However, the low concentration in ferrous ion does not modify the balance between FapyGua and 8-oxodGuo. On the contrary, in the presence of a larger amount of iron (100 μM), the formation of FapyGua increases at the expense of 8-oxodGuo. This may be explained by a significant reduction of the 8-hydroxy-7,8-dihydroguan-7-yl precursor into FapyGua. To assess the effect of iron on the formation of tandem lesions irrespective of the yield of hydroxyl radicals, the ratio between the rate of formation of the latter damage versus 8-oxodGuo was calculated (Figure 6). The relative amount of 8-oxodGuo/dβF increased in the presence of iron in γ-irradiated DNA solutions. In addition, the ratio obtained in the Fenton experiments was higher than that measured upon exposure of DNA to γ-radiation in the absence of ferrous ions. Thus, it clearly appears that iron is involved in the mechanism of formation of tandem base damage (vide infra). The presence of tandem lesions in γ-irradiated samples in which no iron was added can be partly explained by the presence of small amounts of contaminating metal ions in calf thymus DNA (0.75 μM as determined by atomic absorption).

Photoexcited riboflavin was not able to induce any detectable amount of 8-oxodGuo/dβF tandem lesions. Photosensitized one-electron oxidation of the guanine base leads to the formation of the related purine radical cation. The latter species further undergoes hydration,[25−27] yielding the reducing 8-hydroxy-7,8-dihydroguan-7-yl radical also obtained upon addition of hydroxyl radical at the C-8 position. This process is the main base degradation pathway within type I photosensitized DNA since guanine exhibits the lowest ionization potential.[28] Thus, the absence of tandem lesions in riboflavin-photosensitized DNA clearly shows that the initial radical event involved in the formation of 8-oxodGuo and dβF tandem lesion does not implicate the guanine moiety. Experiments using rose bengal as a type II photosensitizer also show that guanine 4−8 endoperoxide,[29] the initial singlet oxygen oxidation product, is not a precursor of 8-oxodGuo and dβF tandem lesion.

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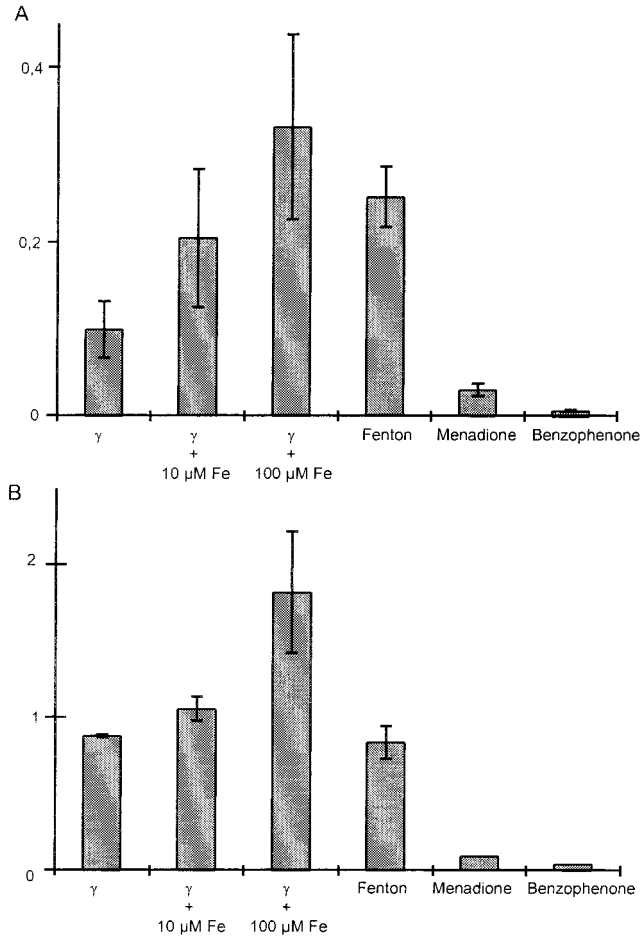

Figure 6. Relative yield of formation of the dβF/8-oxodGuo tandem lesions (A) and the thymine degradation products (5-FordUrd and 5-HMdUrd) (B) with respect to single 8-oxodGuo damage (determined following phosphodiesterase digestion) from calf thymus DNA upon γ-irradiation, γ-irradiation in the presence of increasing amounts of iron, Fenton reaction, menadione, and benzophenone photosensitization.

The formation of several thymine degradation products was also monitored by HPLC-ESI-MS/MS. Thus, the levels of 5-formyl-2′-deoxyuridine (5-FordUrd) and 5-(hydroxymethyl)-2′-deoxyuridine (5-HMdUrd) were measured within calf thymus DNA samples previously hydrolyzed using nuclease P1 and alkaline phosphatase (details on the latter assay will be published elsewhere). The two thymine modifications were detected in γ-irradiated aerated aqueous solutions of isolated DNA in either the presence or absence of iron, and in the Fenton reaction sample. The rates of formation were found to increase linearly with the dose as previously described in isolated DNA.[30,31] However, 5-FordUrd and 5-HMdUrd were not generated at least in detectable amount in aqueous aerated solution of DNA photosensitized with either riboflavin or rose bengal in which dβF and 8-oxodGuo tandem lesions were not detected. In contrast, tandem lesions were shown to be generated in low amounts within DNA photosensitized by menadione and to a lesser extent by benzophenone (Figure 6). Interestingly, the two latter photosensitizers, acting mainly through a type I mechanism, have been reported to yield higher thymine damage than riboflavin.[32] Altogether, these observations showed that methyl oxidized thymidine nucleosides and tandem lesions are generated in a roughly constant ratio within oxidized DNA (Figure 6). As a conclusion, these results strongly suggest that pyrimidine bases are the initial targets involved in the $^{\bullet}$OH-mediated formation of dβF and 8-oxodGuo tandem lesions.[5,33,34]

The present observations allow refinement of the mechanism of formation of the dβF and 8-oxodGuo tandem lesions proposed by Box et al.[5] (Figure 7). The 6(5)-hydroxy-5(6)-dihydrothym-5(6)-yl radical (**1**), produced by addition of hydroxyl radical to the thymine moiety, is converted, in the presence of molecular oxygen, into the related peroxyl radical **2**. In a subsequent step, intramolecular electron transfer leads to the reduction of the latter peroxyl radicals, which after protonation gives rise to the related hydroperoxides on one hand. Oxidation of the vicinal guanine moiety into a radical cation **3** is expected on the other hand. Support for the latter reaction is provided by the observation that alkylperoxyl radicals produced upon thermal degradation of 2,2′-azobis(2-methylpropionamidine) efficiently oxidize guanine residues within DNA.[35] Following hydration, the guanine radical cation would yield the 8-hydroxy-7,8-dihydroguan-7-yl reducing radical **4** which is further oxidized into 8-oxoGua (**6**).[25] Iron is likely to be involved in the fate of the 6(5)-hydroperoxy-5(6)-hydroxy-5,6-dihydrothymine moiety. Indeed, the latter hydroperoxydes are likely to be converted into the highly reactive 6(5)-oxyl radical (**5**) via an organic Fenton reaction. At last, a β-scission reaction is likely to account for the pyrimidine ring opening and subsequent fragmentation, leading to the formylamine remnant (**7**). This may explain the strong effect of iron II on the formation yield of tandem lesions.

Moreover, the postulated mechanism of formation of 8-oxodGuo/dβF could also account for the generation of other tandem base damage. Indeed, the entity carrying 6(5)-hydroperoxy-5(6)-hydroxy-5,6-dihydrothymidine and the guanine radical cation can be the precursor of imidazolone and oxazolone. As well, 5,6-dihydroxy-5,6-dihydrothymidine and 5-hydroxy-5-methylhydantoin can be generated from the 6(5)-hydroperoxy-5(6)-hydroxy-5,6-dihydrothymidine. In addition, evidence was obtained for the formation of dβF/8-oxodGuo tandem base damage deriving from irradiated CpG and GpC sequences.[9,36] The similarity of the reactivity of radicals arising from the addition of hydroxyl radical to thymine and cytosine allows the mechanism presented above to be extended to the latter base. *N*-(2-Deoxy-β-δ-*erythro*-pentofuranosyl)formylamine might also not be the only degraded product−from cytosine that can be involved in tandem base lesions. Further work is required to explore the complete chemistry of tandem base lesions in DNA.

Conclusion

The method used for the specific detection of both dβF-8-oxodGuo and 8-oxodGuo-dβF tandem lesions simultaneously is highly sensitive, with a detection limit close to 10 fmol. A mechanism for the formation of the tandem base damage was proposed. In addition, the present work suggests that 8-oxodGuo/dβF adducts are not the only significant form of tandem damage in DNA subject to oxidative stress. Further work aimed at characterizing new multiple DNA base damage is in progress.

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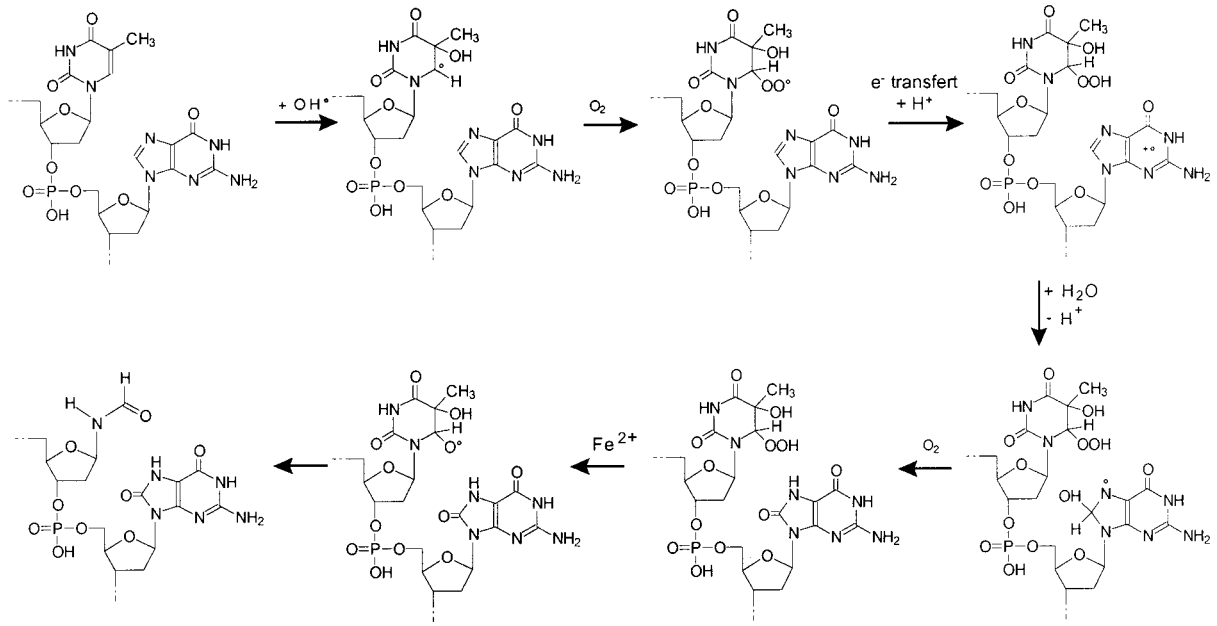

Figure 7. Proposed mechanisms for the formation of the dβF/8-oxodGuo tandem lesions.

Experimental Section

Chemicals. Nuclease micrococcal, Nuclease P1 (*penicillium citrium*), alkaline phosphatase, H_2O_2, rose bengal, menadione, and calf thymus DNA were obtained from Sigma (St Louis, MO). Calf spleen phosphodiesterase and snake venom phophodiesterase were purchased from Boehringer Mannheim (Mannheim, Germany). Ethylenediamine tetraacetic acid was purchased from Interchim (Montluçon, France). Riboflavin (7,8-dimethyl-10-(*δ-ribo*-2,3,4,5-tetrahydroxypentyl)isoalloxazine) was obtained from BDH Chemicals (Poole, UK). $FeSO_4$ was obtained from Merck (Darmstadt, Germany). Benzophenone was purchased from Prolabo (Paris, France).

Water was deionized with a Millipore-Milli-Q system. Oligodeoxynucleotides, 8-oxodGuo, 5-FordUrd, and 5-HMdUrd were synthesized as previously described.[18,37]

Preparation and Calibration of Tandem Base Damage Standards. Modified oligodeoxynucleotides d(AFβG°C), d(AFαG°C), d(AG°FβC), d(AG°FαC), d(CAGTACGTAGGAGFβG°CCATCGATAG), and d(CAGTACGTAGGAG°FβGCCATCGATAG) (2 $AU_{260\,nm}$) were digested into d(FβG°C), d(G°FβC), d(FαG°C), d(G°FαC), d(FβG°CCATCGATAG), and (G°FβGCCATCGATAG), respectively, by incubation for 1.5 h at 37 °C with 10^{-2} U of calf spleen phosphodiesterase (2 U/mL) in 50 μL of 0.02 M ammonium citrate. Then, 3×10^{-3} U of snake venom phosphodiesterase in buffer that contained 500 mM Tris, 1 mM EDTA, pH 8.5 (10 μL), was added. The resulting mixture was incubated for 10 min at 37 °C and the enzymatic reaction was quenched by heating at 90 °C for 3 min. The resulting samples were analyzed and purified by HPLC on a 5 μm C_{18} (250 × 4.6 mm, Hypersil) column using a 0−10% linear gradient of CH_3CN in 25 mM ammonium formiate (retention time Rt = 17.2 and 18.4 for dβF-8-oxodGuo and 8-oxodGuo-dβF, respectively). dβF-8-oxodGuo (ESI-MS) *m/z* 505.2 $[M - H]^-$; UV (H_2O, pH 7) λ_{max} 246 and 296. 8-oxodGuo-dβF (ESI-MS) *m/z* 505.1 $[M - H]^-$; UV (H_2O, pH 7) λ_{max} 246 and 296.

Calibrations of the solution were made by measuring the absorbance at 293 nm. The concentrations were determined using the molecular absorption coefficient of 8-oxo-7,8-dihydro-2′-deoxyguanosine (ϵ_{293} = 9700). Calibration of the solution of 8-oxodGuo-dβF was assessed by the HPLC-EC measurement of the level of 8-oxodGuo generated upon enzymatic hydrolysis of the dinucleoside monophosphate with nuclease P1 and phosphatase alkaline.

HPLC-ESI-MS/MS Detection of the dβF/8-oxodGuo Tandem Base Damage. The HPLC apparatus consisted of a 7100 Hitachi-Merck pump (Merck, Darmstadt, Germany) connected to a SIL-9 automatic injector (Shimadzu, Tokyo, Japan) and equipped with an Uptisphere ODB (5 μm, 150 × 2 mm i.d.) octadecylsilyl silica gel column (Interchim, Montluçon, France). Elution was performed using a 8−20% linear gradient of MeOH/5 mM ammonium formiate buffer over a period of 5 min and maintained for 10 min at 20% of MeOH/5 mM ammonium formate buffer with a 0.2 mL/min flow rate. Methanol was added at the outlet of the column and prior to the inlet of the mass spectrometer at a flow rate of 0.2 mL/min. Under the present HPLC conditions, the retention times for dβF-8-oxodGuo and 8-oxodGuo-dβF were 5.9 and 6.7 min, respectively. The API 3000 spectrometer (Perkin-Elmer/SCIEX, Toronto, Canada) was operated in the negative mode using the multiple reaction monitoring technique (MRM). Two transitions were monitored: 505 → 362 and 505 → 166. The dwell time was 1500 ms for both signals. Calibration of the response of the spectrometer was obtained by injecting increasing amounts of a mixture of the two isomeric dβF and 8-oxodGuo tandem lesion standards prior and after each series of injections. To control the stability of the signal, injections of 1 pmol of the two tandem lesions were randomly performed in the series of analyses.

γ-Irradiation of Isolated DNA. An aqueous aerated solution of calf thymus DNA (0.85 mg·mL^{-1}) was exposed to the γ-rays of a ^{60}Co source immerged in a pool. The dose rate was 20 Gy·min^{-1} as determined by the poly(methyl methacrylate) dosimetry. A continuous air bubbling was maintained during the irradiation. Periods of irradiation ranged from 0 to 5 min. Additional irradiation experiments were also carried out in the presence of iron(II) and EDTA. A freshly made aqueous solution consisting of $FeSO_4$ (10 mM) and EDTA (10 mM) was added to the DNA sample prior to γ-irradiation. Typically, the irradiations were carried out with 1 mL of aqueous solution of calf thymus DNA to which were added 0, 1, or 10 μL of the Fe/EDTA aqueous solutions (final iron concentration 0, 10, and 100 μM). At the end of the reaction, calf thymus DNA was precipitated once with 3 M sodium acetate:ethanol (1:4 v/v) and redissolved in water (1 mL).

DNA Photosensitization. Aqueous aerated solutions of calf thymus DNA (1 mL of a 0.85 mg/mL solution) were exposed to either UV-A in the presence of riboflavin, menadione, and benzophenone or visible light in the presence of rose bengal. The UV-A light was provided by 350 nm lamps (4 × 15 W) of a Rayonet photoreactor (The Southern New England Ultraviolet Company, Hamden, CT) whereas the visible light was generated by a halogen lamp (500 W). The concentration of the photosensitizers was one-tenth of the saturation for riboflavin (ca. 0.05 mM, absorption at 350 nm, A_{350}: 0.13), 1 mM for menadione (A_{350}: 2.9), and at saturation for benzophenone (A_{350}: 0.02). The

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absorption of the rose bengal containing solution was 1 AU at 550 nm. The defined periods of irradiation were 0, 2, 5, and 10 min for both riboflavin and rose bengal. For the latter photosensitizer, additional exposures of 30 min were carried out. For menadione and benzophenone, times of exposure of 0, 30, 60, and 120 min were used. Photosensitization experiments were performed in a glass vial under permanent air bubbling to maintain the solution saturated with oxygen. At the end of the reaction, calf thymus DNA was precipated once with 3 M sodium acetate/ethanol (1:4 v/v) and redissolved in water (1 mL).

Fenton Reaction. Aerated solutions of calf thymus DNA (1 mL) were incubated for 30 min at 37 °C in the presence of freshly prepared 10 μM Fe^{2+}/EDTA [1/1] and 0, 10, 50, or 100 μM hydrogen peroxide. DNA was then precipitated as described above.

DNA Analysis. Aliquot fractions of DNA samples (100 μL, 85.5 μg) were first incubated with 15 μL micrococcal nuclease (0.75 U) and 30 μL of buffer (200 mM succinic acid, 100 mM $CaCl_2$, 0.2 M ammonium citrate, pH 5) for 30 min at 37 °C. The enzymatic reaction was quenched by heating at 90 °C for 3 min. The resulting solution was incubated with 1.2×10^{-2} U of calf spleen phosphodiesterase (2 U/mL) for 3 h at 37 °C. Then, 20 μL of buffer (500 mM TRIS, 1 mM EDTA, pH 8.5) that contained 20 U of alkaline phosphatase was added and the incubation was resumed for 1.5 h. Finally, 1.8×10^{-3} U of snake venom phosphodiesterase was added. The enzymatic reaction was pursued for 15 min at 37 °C prior to being quenched upon heating at 90 °C for 3 min. Chloroform (50 μL) was added and the resulting solution was centrifuged (1500 g). Subsequently, the aqueous layer was collected, concentrated under vacuum, and transferred into HPLC injection vials. An aliquot fraction of the sample (5 μL) was diluted 10-fold in water and the resulting solution was analyzed by HPLC with UV and EC detection to determine the amount of DNA and 8-oxodGuo, respectively.

Nuclease P1 and Alkaline Phosphatase Hydrolysis. Aliquot fractions of DNA solution (100 μL, 85.5 μg) were digested upon incubation for 2 h at 37 °C with 10 U of nuclease P1 in an aqueous solution of 30 mM NaOAc and 0.1 mM $ZnSO_4$, pH 5.5, in a total volume of 110 μL. Then, dephosphorylation of the resulting nucleotides was achieved by addition of 11 μL of alkaline phosphatase buffer that contained 500 mM Tris, 1 mM EDTA (pH 8.5), and 10 U of alkaline phophatase. The mixture was then incubated for 2 h at 37 °C. Samples were transferred into HPLC injection vials. An aliquot fraction of the sample (5 μL) was diluted 10-fold with water. Then, the resulting solution was analyzed by HPLC with UV and EC detections to determine the amount of DNA and 8-oxodGuo, respectively.

HPLC-EC Detection of 8-oxo-dGuo. The HPLC-EC system consisted of a model 2150 LKB pump (Pharmacia LKB Biotechnology, Uppsala, Sweden) connected to a SIL-9A autosampler (Shimadzu, Kyoto, Japan) equipped with a Uptisphere ODSB (5 μm, 250 × 4.6 mm) octadodecylsilyl silica gel (Interchim, Montluçon, France). The isocratic eluent was a 50 mM aqueous solution of potassium dihydrogen phosphate (pH 5.5) that contained 9% MeOH. Under these conditions, the retention time of 8-oxodGuo was 17 min. Coulometric detection was provided by a Coulochem II detector equipped with a 5010 cell (Esa, Chelmsford, MA). The respective potentials of the two electrodes were set at 200 and 400 mV. Elution of unmodified nucleosides was simultaneously monitored by a Waters 484 UV variable wavelength spectrophotometer set at 280 nm. Both EC and UV signals were collected on a D7500 Hitachi integrator (Tokyo, Japan).

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