Efficiency of Excision of 8-Oxo-guanine within DNA Clustered Damage by XRS5 Nuclear Extracts and Purified Human OGG1 Protein

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Received June 14, 2001; Revised Manuscript Received July 26, 2001

ABSTRACT: A major DNA lesion is the strongly mutagenic 8-oxo-7,8-dihydroguanine (8-oxoG) base, formed by oxidative attack at guanine and which leads to a high level of G•C→T•A transversions. Clustered DNA damages are formed in DNA following exposure to ionizing radiation or radiomimetic anticancer agents and are thought to be biologically severe. The presence of 8-oxoG within clustered DNA damage may present a challenge to the repair machinery of the cell, if the OGG1 DNA glycosylase/AP lyase protein, present in eukaryotic cells, does not efficiently excise its substrate, 8-oxoG. In this study, specific oligonucleotide constructs containing an 8-oxoG located in several positions opposite to another damage (5,6-dihydrothymine (DHT), uracil, 8-oxoG, AP site, or various types of single strand breaks) were used to determine the relative efficiency of purified human OGG1 and mammalian XRS5 nuclear extracts to excise 8-oxoG from clustered damages. A base damage (DHT, uracil, and 8-oxoG) on the opposite strand has little or no influence on the rate of excision of 8-oxoG whereas the presence of either an AP site or various types of single strand breaks has a strong inhibitory effect on the formation of a SSB due to the excision of 8-oxoG by both hOGG1 and the nuclear extract. The binding of hOGG1 to 8-oxoG is not significantly affected by the presence of a neighboring lesion.

Ionizing radiation as well as radiomimetic anticancer agents such as bleomycin induce damage in DNA such as single lesions, tandem base damage (*1*−*3*), or clustered DNA damage, in which two or more elemental lesions are induced within one to two helical turns of DNA (*4*, *5*). The complexity of clustered DNA damage correlates with the ionization energy of the radiation track (*6*) and clustered damages are thought to compromise the repair machinery of the cell. Indeed, for high linear energy transfer (LET)[1] radiation, the yield of clustered damage is estimated to be high, with more than 50% of single (SSB) and double strand breaks (DSB) having neighboring lesions (*7*, *8*). High-LET radiations have some consequences on health since about 10−15% of lung cancers can be attributed to radon (*9*). The transformation potential and the viability of the cells depend on the kinetics of processing of clustered damages that comprise either several damaged bases or base lesions associated with abasic sites (AP), SSB or DSB, formed within a few base pairs of each other. The concomitant excision of two opposed lesions would convert a clustered damage into a DSB, which, if unrepaired, may be lethal for the cell, whereas the sequential excision and repair of one lesion of the two closely opposed lesions may avoid formation of a DSB.

Only few recent studies have focused on the excision of clustered damage (*10*−*14*), which contain two lesions, one on each strand, or tandem base lesions (*15*, *16*) by purified proteins. A SSB or AP site has a major attenuation on the rate of excision of a base lesion by prokaryotic enzymes and the eukaryotic enzyme yeast OGG1 (*17*). The only study with nuclear cell extracts showed that the greatest inhibitory effect of excision of a base modification [5,6-dihydrothymine (DHT)] is the presence of an AP site on the complementary strand within 5 base pairs (*13*). To date, the influence of the presence of an opposite SSB on the excision of a base lesion by nuclear extracts has not been investigated. In the present study, we have concentrated on the effect of either an AP site (normal or reduced) or SSBs with various termini on the excision of 8-oxo-7,8-dihydroguanine (8-oxoG), a major oxidative and radiation-induced damage, by human OGG1 DNA glycosylase/AP lyase (hOGG1) (*18*−*24*). For comparison with hOGG1, XRS5 nuclear extracts (obtained from a Ku deficient CHO cell line) (*25*) have been used as a model of a mixture of the various proteins that may excise clustered DNA damage within the nucleus.

The hOGG1 protein is a 38 kDa (345 amino acids) protein with a helix-hairpin-helix (HhH) motif in its catalytic domain that shares homology with that of the Nth (endonuclease III) protein. Despite the fact that both the eukaryotic OGG1 and the bacterial formamidopyrimidine DNA glycosylase (Fpg)

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[1] Abbreviations: DHT, 5,6-dihydrothymine; AP, apurinic/apyrimidic site; 8-oxoG, 8-oxo-7,8-dihydroguanine; LET, linear energy transfer; bp, base pair; SSB, single strand break; DSB, double strand break; BER, base excision repair; hOGG1, human OGG1 DNA glycosylase/AP lyase; Fpg, formamidopyrimidine DNA glycosylase; Nth, endonuclease III; XRS, X-ray sensitive; PMSF, phenylmethanesulfonyl fluoride; CHO, Chinese hamster ovary.

10.1021/bi0112356 CCC: $20.00
Published on Web 09/05/2001

Table 1: Sequences of the Oligonucleotides[a]

Position	Sequence	Strand
-5	5′-ctcttagtca ggaa**Y**atgtc tctatgctgg gagcaaaggc-3′	(1)
	3′-gagaatcagt ccttNtaca**X** agatacgacc ctcgtttccg-5′	(2)
-3	5′-ctcttagtca ggaata**Y**gtc tctatgctgg gagcaaaggc-3′	
	3′-gagaatcagt ccttatNca**X** agatacgacc ctcgtttccg-5′	
-1	5′-ctcttagtca ggaatatg**Y**c tctatgctgg gagcaaaggc-3′	
	3′-gagaatcagt ccttatacN**X** agatacgacc ctcgtttccg-5′	
+1	5′-ctcttagtca ggaatatgtc **Y**ctatgctgg gagcaaaggc-3′	
	3′-gagaatcagt ccttataca**X** Ngatacgacc ctcgtttccg-5′	
+3	5′-ctcttagtca ggaatatgtc tc**Y**atgctgg gagcaaaggc-3′	
	3′-gagaatcagt ccttataca**X** agNtacgacc ctcgtttccg-5′	
+5	5′-ctcttagtca ggaatatgtc tcta**Y**gctgg gagcaaaggc-3′	
	3′-gagaatcagt ccttataca**X** agatNcgacc ctcgtttccg-5′	

[a] X = 8-oxoG, Y = either DHT, 8-oxoG, uracil, AP, reduced AP site, β-SSB, β-δ-SSB, HAP1-SSB or the normal corresponding base (T for DHT, uracil for AP and rAP sites, G for 8-oxoG). N = Normal base complementary to the **Y** base (A opposite to uracil, AP or DHT, C opposite to 8-oxoG). **−5** up to **−1** = position of the **X** base 5′ from the **Y** base, on the complementary strand. **+1** up to **+5** = position of the **X** base 3′ from the **Y** base, on the complementary strand.

protein excise 8-oxoG from the DNA, they do not contain any sequence homology. The yOGG1 and hOGG1 proteins have similar abilities to remove 8-oxoG, formamidopyrimidine, methyl formamidopyrimidines, and AP site (particularly those opposite cytosine) (*26*) and possibly 8-oxo-7,8-dihydroadenine, when opposite to cytosine or methylcytosine (*27*), from DNA but not other types of oxidatively induced or radiation-induced damages in DNA. Similar to Nth, the AP lyase activity of the OGG1 protein proceeds by a β-elimination, producing a SSB with 5′-phosphate and 3′-phospho-α,β unsaturated aldehyde termini (*28*, *29*). In a number of human diseases, cell lines such as KG-1 leukemic cell may contain mutant hOGG1 protein (*30*−*38*).

In this report, specific oligonucleotides have been constructed containing 8-oxoG at precisely known positions opposite to a second lesion (DHT, AP site, reduced AP site, or a variety of SSBs) on the complementary strand. The influence of the presence of the second lesion within the clustered damage on the efficiency of excision of the highly mutagenic 8-oxoG by hOGG1 as well as by the XRS5 mammalian nuclear extracts has been assessed. The kinetics constants (K_M, k_{cat}, and specificity constants) have been determined for the excision of 8-oxoG within various types of clustered damages by hOGG1. Binding experiments were also undertaken to assess whether the presence of an opposite lesion influences the recognition process, the catalytic excision step, or both processes.

MATERIALS AND METHODS

Substrate Oligonucleotides. All oligonucleotides were purchased from Genosys or Glen Research. The sequences of the various oligonucleotides are presented in Table 1. Strand 1 contains either DHT, 8-oxoG, uracil, an AP site, various SSBs, or the corresponding undamaged base, as controls, at the variable position **Y**. Strand 2, containing 8-oxoG at the fixed position **X**, was 5′-end labeled with ^{32}P by incubation with 10 units of T4 polynucleotide kinase (GIBCO BRL) and 50 μCi [γ-^{32}P]ATP (6000 Ci/mmol, 10 mCi/mL, NEN Dupont) for 1 h in 25 μL of the recommended buffer at 37 °C. Following purification of the single-stranded oligonucleotide on a 12% denaturing polyacrylamide gel, the labeled oligonucleotide was hybridized with 1.5-fold excess of the various purified, nonradiolabeled complementary strands. The solutions for the hybridization reaction were heated to 80 °C for a few minutes and then allowed to cool slowly to room temperature over ∼2 h. The double-stranded oligonucleotides were then purified in a native 10% polyacrylamide gel, run for 3 h at 300 V in TBE (1×). All the oligonucleotides subsequently prepared were gel purified as described above, unless otherwise stated. To prepare the oligonucleotides containing an abasic site at given positions, the ^{32}P labeled double-stranded oligonucleotides containing a uracil were treated at 37 °C with 1 unit of uracil-DNA-glycosylase (UDG) (GIBCO-BRL) in 50 μL of buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA] for 30 min. The efficiency of formation of an AP site is >98% as verified by treatment with Nth as described previously (*14*, *17*).

Purified Proteins. The purified hOGG1 protein was extracted and purified as described in ref *36*. The purified Nth and HAP1 proteins were generous gifts from Prof. Rick Wood (Imperial Cancer Research Fund, South Mimms, U.K.) (*39*) and Prof. Ian Hickson (Institute of Molecular Medicine, Oxford, U.K.) (*40*), respectively.

Preparation of XRS5 Nuclear Extracts.. The nuclear extracts were prepared as previously described (*13*) from a Ku deficient CHO-derived cell line, XRS5 (*25*), to avoid possible interference by Ku binding to termini of linear DNA (*41*). Briefly, the cells grown in exponential phase were harvested by centrifugation at 1000*g* for 10 min at 4 °C. The resulting pellet was washed twice in 30 vol of PBS and then resuspended in an equal volume of buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) and incubated on ice for 15 min. After rupture of the cytoplasmic membrane by drawing several times into a 0.5 μm diameter needle, the cells were centrifuged at 12000*g* and the nuclear pellet resuspended in 2/3 vol of buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF) for 30 min with agitation on ice. After centrifugation, the supernatant was dialyzed against buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol containing 0.5 mM DTT,

0.5 mM PMSF) and stored at −80 °C. Several extract preparations were prepared and then pooled. This pooled batch, which was used throughout these experiments, showed insignificant variation of its activity with storage time.

Preparation of a Reduced-AP Site, a β-SSB, and a HAPI-SSB. A reduced AP site was prepared using $NaBH_4$ (Sigma) and purified as described previously (*17*, *42*). The efficiency of reduction was checked by labeling both strands of the oligonucleotides prior to an incubation with either endonuclease III, Fpg, or OGG1 proteins which do not cleave effectively a reduced AP site as compared with their ability to cleave a nonreduced AP site as control. A β-SSB, with 5′-phosphate and 3′-phospho-α,β unsaturated aldehyde termini, and a HAP-1 SSB, with 5′-phosphate and 3′-OH termini, were prepared by treatment of an oligonucleotides containing an AP site, respectively, with 10 ng of endonuclease III protein or with 500 pg of human AP endonuclease 1 (HAP1), as previously described (*14*, *17*). The incision of an AP site by endonuclease III is not affected by the presence of an opposite 8-oxoG at any of the tested positions (*13*). The oligonucleotides were then precipitated by addition of cold ethanol, washed, dried, and resuspended in the appropriate amount of TE buffer supplemented with 100 mM NaCl. The efficiency of formation of a β-SSB or HAP1-SSB is >98% (data not shown), as verified from the migration profiles of the labeled oligonucleotides following PAGE. To obtain a probe containing a β-δ-SSB with 3′ and 5′ phosphate termini, two oligonucleotides were used to form strand 1 with a SSB at position **Y**. The oligonucleotide 5′ of the SSB at position **Y** contains a 3′-phosphate terminus whereas the 3′ oligonucleotide contains a 5′-phosphate terminus.

Cleavage Assays for SSB Analysis. The double-stranded oligonucleotides (10000 cpm, 200 fmol) were incubated with the known amounts of either hOGG1 or XRS5 nuclear extracts, as specified in the legends of the figures, in 5 μL of the incubation buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA) at 37 °C for 30 min. Subsequently, 5 μL of the denaturing stop solution (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 2 mM EDTA, pH 8.0) was added to the samples which were then subjected to electrophoresis on a 12% denaturing-PAGE containing 8 M urea in 1× TBE at 85 W for 90 min as described in ref *13*. The dried gel was then exposed to a Bio-Rad PhosphorImager screen to visualize cleaved and full-length DNA fragments using phosphorimagery (Biorad, Molecular Imager FX). Quantification was undertaken using Quantity One software (Bio-Rad) to determine the excision efficiency of each protein for each of the single or clustered damages used. The activity of the labeled, cleaved strand is expressed as a percentage of the total activity of the cleaved and intact strands. The efficiencies for excision of a base lesion within clustered damages are compared with that for excision of a single lesion in the control oligonucleotide at the same concentration of hOGG1 or extract, to assess the effect of the second lesion, present on the unlabeled strand, on excision of the lesion on the labeled strand. The errors represent standard errors of the mean from at least three repeat experiments.

Kinetic Measurements. For kinetic measurements, known concentrations of the oligonucleotides (1−50 nM) containing either a single 8-oxoG damage as control (C) or an 8-oxoG 1, 3, or 5 bases 5′ or 3′ to an AP site or a β-δ-SSB on the complementary strand were incubated for 10 min at 37 °C with a known concentration of hOGG1 (2−4 nM). The concentration of the cleaved, labeled strand relative to that of the intact strand was determined as described above for the SSB assay with denaturing PAGE. The initial reaction rate, obtained from the concentration of cleaved DNA per minute for a constant concentration of protein, was plotted versus the substrate concentration using a double reciprocal dependence based on Michaelis−Menten kinetics. Each point represents the standard error of the mean for at least three repeated experiments using six different concentrations of the substrates. The values of K_M and V_{max} were obtained from the linear regression analysis of the Lineweaver−Burk plots, using the method of weighted least squares, the ordinate of each point being weighted by its reciprocal.

Electromobility Shift Assays (EMSA). To assess the ability of hOGG1 to bind to oligonucleotides containing 8-oxoG opposite to an AP site at various positions, binding reactions were performed by mixing 5′-^{32}P-end-labeled oligonucleotides (10 000 cpm, 0.5 nM) with 5 μL of binding buffer (20 mM HEPES, pH 7.9, 100 mM in KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.2 mM EDTA, 20% glycerol) and incubating for 15 min at 4 °C. The samples were then loaded directly onto a 5% polyacrylamide gel. After electrophoresis at 35 mA in TAE 0.7× (4.7 mM Tris-HCl, 2.3 mM sodium acetate, 0.7 mM EDTA, pH 7.9) for 2 h, the gels were dried, exposed to a phosphorimager screen and quantified as described above.

RESULTS

Effect of a Neighboring Base Lesion or AP Site on the Excision of 8-oxoG by XRS5 Nuclear Extracts. The influence of a neighboring lesion on the efficiency of excision of the highly mutagenic 8-oxoG base lesion by XRS5 nuclear extracts was determined using the oligonucleotides described in Table 1 that contain a single 8-oxoG (control C) or an 8-oxoG lesion near to either DHT, uracil, a second 8-oxoG, or an AP site at positions −5 to +5 on the complementary strand (Figure 1). The main inhibitory effect on excision of 8-oxoG was seen in the presence of an AP site on the complementary strand (Figure 1C), whereas the presence of a neighboring base lesion has little or no effect on the excision of 8-oxoG by the nuclear extract. Since the presence of a uracil has little or no effect on the excision of 8-oxoG compared with that seen with an AP site at the corresponding positions, it is inferred that few uracil moieties are converted into AP sites by UDG protein, presumably present in the extract, during the incubation time, when significant levels of 8-oxoG are excised by the extract. This difference was verified from the efficiencies of cleavage of either uracil, 8-oxoG, or an AP site, as a single lesion, by XRS5 nuclear extracts as shown in Figure 2. The efficiency of cleavage of a single lesion from the oligonucleotides by the nuclear extract increases in the order AP site > 8-oxoG > uracil. Since the rate of conversion of an AP site into a SSB by the proteins in the nuclear extract is greater than that of 8-oxoG, the inhibitory effect of a neighboring AP site on the excision of 8-oxoG by the nuclear extracts may reflect the presence of a SSB opposite to 8-oxoG.

The effect of a reduced AP site, which is refractory to cleavage by AP lyases present in the extract, on the excision

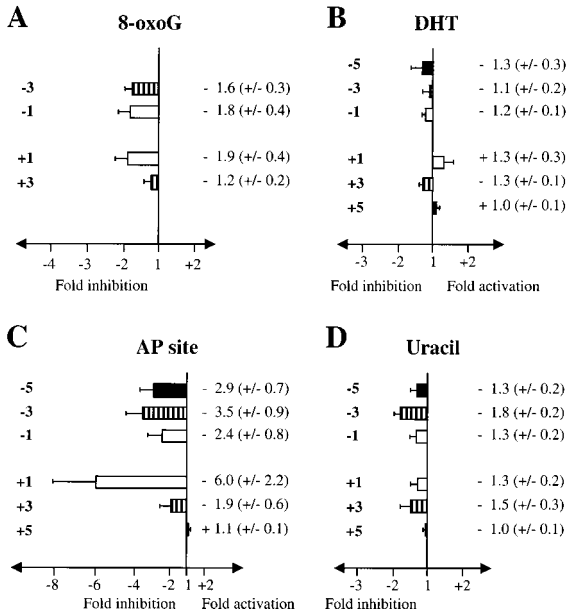

FIGURE 1: Effect of a neighboring lesion, 8-oxoG (A), DHT (B), AP site (C), and uracil (D), at the different positions on the efficiency of excision of 8-oxoG by XRS5 nuclear extracts (2.5−10 μg), expressed as fold inhibition/activation by comparison with the control oligonucleotide containing an 8-oxoG lesion. The error bars represent the standard deviation from three to five different experiments.

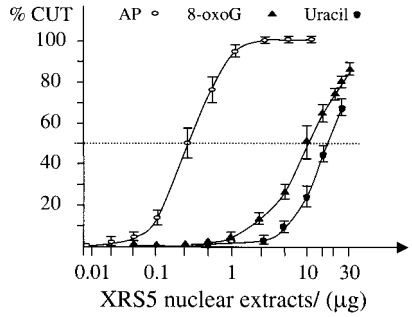

FIGURE 2: The dependence of efficiency of excision of either an AP site (○), 8-oxoG (▲), or uracil (●) on the concentration of XRS5 nuclear extract from oligonucleotides containing the lesion on the ^{32}P-labeled strand. The graphs represent the amount of cleaved DNA as a percentage of the total amount of DNA. The error bars represent the standard deviation from three different experiments.

of 8-oxoG on the complementary strand has a large inhibitory effect on the excision of 8-oxoG by the extract (Figure 3A). However, the extracts do cleave the reduced AP site-containing oligonucleotide with a slightly greater efficiency than that for a nonreduced AP site (Figure 3B) but with significantly greater efficiency than that for 8-oxoG. The inhibition of excision of 8-oxoG by a reduced AP site could be due to the conversion of the reduced AP site into a SSB by a protein(s) present in the XRS5 nuclear extract. Therefore, the following experiments were undertaken to assess the inhibitory effect of a SSB on the excision of 8-oxoG on the complementary strand by the nuclear extracts.

Effect of a SSB on the Excision of 8-oxoG by XRS5 Nuclear Extracts. Various SSBs (β-SSB, HAP1-SSB, and β-δ-SSB with different break termini as shown in Figure 4) were used to determine their inhibitory effects on excision of 8-oxoG within oligonucleotides by nuclear extracts. SSB induced in DNA by ionizing radiation or reactive oxygen species represent a loss of a nucleotide where the resulting SSB

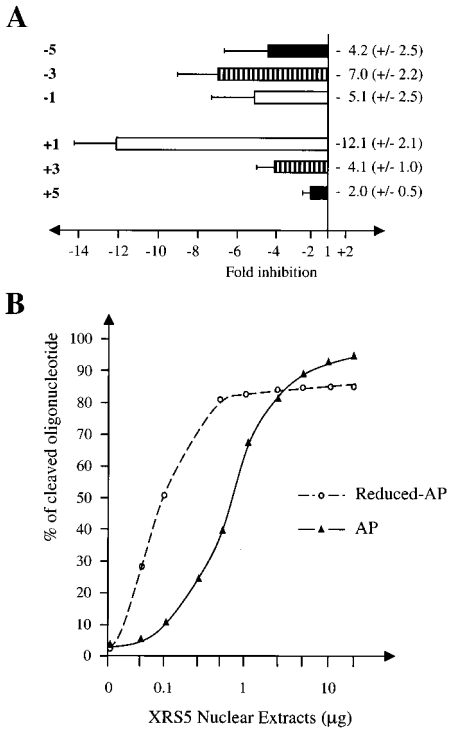

FIGURE 3: (A) Effect of a neighboring reduced AP site at the different positions on the efficiency of excision of 8-oxoG by XRS5 nuclear extracts (2.5−10 μg), expressed as fold inhibition by comparison with the control oligonucleotide containing an 8-oxoG lesion. The error bars represent the standard deviation from three different experiments. (B) Comparison of the efficiencies of excision of either an AP site (▲) or a reduced AP site (○) on the concentration of XRS5 nuclear extract from oligonucleotides containing the lesion on the ^{32}P-labeled strand. The graphs represent the amount of cleaved DNA as a percentage of the total amount of DNA. The error bars represent the standard deviation from three different experiments.

termini are a 5′-phosphate and either a 3′-phosphate (see Figure 4C) or a 3′-phosphoglycolate (*43*). A β-SSB, created by β lyase activity of endonuclease III on an AP site, has an inhibitory effect on the excision of 8-oxoG on the complementary strand by the nuclear extract (Figure 4A). The maximum inhibition (4.9-fold) occurs when the β-SSB is positioned 1 base 3′ to the site opposite to 8-oxoG (position +1, Figure 4A). The profile of this inhibitory effect on the inter-lesion separation is similar to that observed for excision of 8-oxoG in the presence of an AP site at the corresponding positions (Figure 1C).

A HAP1-SSB, obtained by treatment of oligonucleotides containing an AP site with HAP1, was investigated to mimic a SSB, which arises from in vivo processing of an AP site by AP endonucleases. The inhibitory effects of a HAP1-SSB on the excision of 8-oxoG by the extract (Figure 4B) shows a similar profile to that observed for an AP site (Figure 1C). It is worth noting that the inhibitory effect seen in the presence of an opposite HAP1-SSB is larger than that seen in the presence of a β-SSB in Figure 4A. It is suggested that not only the relative position of the SSB but also the type of SSB termini may influence the efficiency of excision of 8-oxoG within a clustered damage by the nuclear extract.

A β-δ-SSB with 5′-phosphate and 3′-phophate strand break termini is more effective than either a β-SSB or a HAP1-SSB (Figure 4, panels A and B, respectively) at inhibiting the excision of 8-oxoG by the XRS5 nuclear extract, as

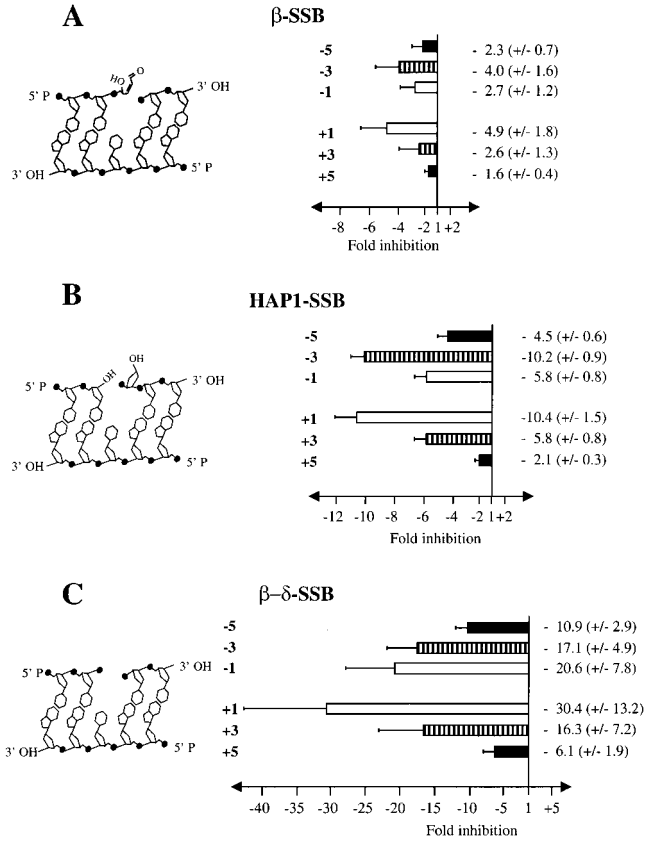

FIGURE 4: Effect of different types of single strand breaks, a β-SSB (A), an HAP1-SSB (B) or a β-δ-SSB (C), at the different positions -5 to $+5$ on the efficiency of excision of 8-oxoG by XRS5 nuclear extracts (2.5−10 μg), expressed as fold inhibition by comparison with the control oligonucleotide containing an 8-oxoG lesion. The error bars represent the standard deviation from four different experiments. The structures of the SSB are shown schematically with an arbitrary choice of base pairs for illustrative purposes and the solid circle (●) represents a phosphate group.

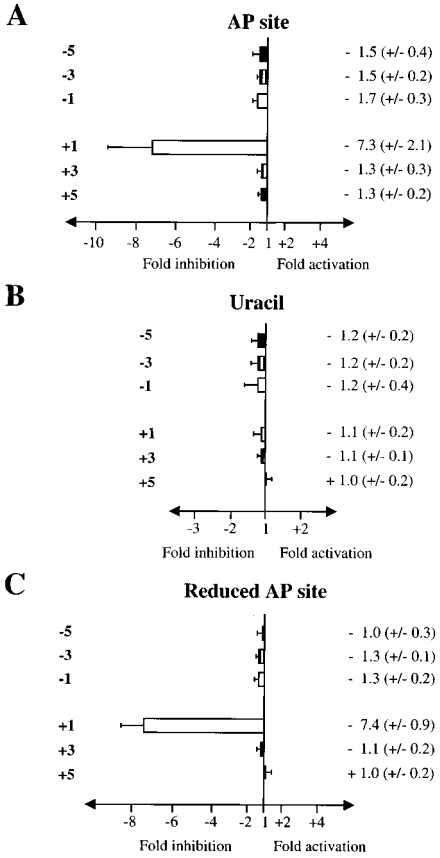

FIGURE 5: Effect of a neighboring lesion, an AP site (A), uracil (B), and a reduced AP site (C) at the different positions -5 to $+5$ on the efficiency of excision of 8-oxoG by hOGG1 (0.25−0.5 ng), expressed as fold inhibition/activation by comparison with the control oligonucleotide containing an 8-oxoG lesion. The error bars represent the standard deviation from five different experiments.

presented in Figure 4C. This inhibitory effect of a β-δ-SSB on the excision of 8-oxoG is also greater (up to 30.4-fold at position $+1$) than that observed with an AP site (up to 6.0-fold, Figure 1C), a reduced AP site (up to 12.1-fold, Figure 2A) or any of the other base lesions tested (Figure 1). The number of base pairs over which the inhibition of excision of 8-oxoG by a neighboring β-δ-SSB occurs, is also larger than that for an AP site.

Effect of a Neighboring Lesion on the Excision of 8-oxoG by the hOGG1 Protein. In mammalian cells, the excision of 8-oxoG from DNA is performed by the OGG1 protein. The purified human OGG1 protein was used to compare the effect of a neighboring damage on the rate of excision of 8-oxoG within a clustered damage by hOGG1 with those reported above with XRS5 nuclear extracts. The presence of either uracil (Figure 5B) or another base lesion [8-oxoG or DHT (data not shown)] does not significantly influence the excision of 8-oxoG by hOGG1, similar to the observations with the yeast OGG1 protein (*17*) and XRS5 nuclear extract. However the presence of an AP site or reduced AP site, particularly at position $+1$ (Figure 5, panels A and C) has an inhibitory effect on the excision of 8-oxoG by hOGG1, similar to that seen with yOGG1 (*17*) but in contrast to the larger effects seen with nuclear extracts.

The presence of either a β-SSB or an HAP1-SSB at position $+1$ (Figure 6, panels A or B, respectively) causes a similar level of inhibition on excision of 8-oxoG by hOGG1. Only the presence of a β-δ-SSB (see Figure 4C) has a larger inhibitory effect on the excision of 8-oxoG by hOGG1, extending over significant inter-lesion distances (Figure 6C). This profile of inhibition with hOGG1 is comparable with that seen using nuclear extracts, incubated with the corresponding oligonucleotides containing a β-δ-SSB and 8-oxoG.

Effect of a Neighboring Lesion on the Kinetic Constants for Excision of 8-oxoG by hOGG1. To gain further insight into the mechanism of the inhibitory effect on incision of an 8-oxoG by hOGG1 in the presence of an AP site or a β-δ-SSB at positions $+5$ to -5, the kinetic constants K_M, k_{cat}, and specificity constants were determined and are presented in Table 2. The major effect on the specificity constant for excision of 8-oxoG is a decrease when an AP site is at position $+1$ or a β-δ-SSB at positions -3 to $+1$, consistent with the corresponding inhibitory effects seen in Figures 5 and 6. This reduction in the specificity constants in the presence of a neighboring AP site at position $+1$ is associated with a large increase in the value of K_M (6-fold increased), whereas the value of k_{cat} decreases slightly. The increased value of K_M for an AP site at position -1 is compensated by an increase of the value of k_{cat}, so that the specificity constant only decreases 2-fold compared with that for the control DNA (C, Table 2). With a β-δ-SSB positioned at positions $+1$ to -3, the specificity constant decreases are accompanied by increases in the values of K_M. The values

Table 2: Kinetic Constants for the Excision of 8-oxoG by hOGG1 in the Presence of Either an AP Site or a β-δ-SSB, at Positions -5 to $+5$ on the Complementary Strand, Determined from Lineweaver−Burk Plots[a]

damage opposite 8-oxoG	[S] (nM)	position	K_M (nM)	k_{cat} (min^{-1}) × 10^{-4}	specificity constant (min^{-1} nM^{-1}) × 10^{-4}
AP site	1−50	−5	5.5 ± 1.8	182 ± 19	33.3 ± 8.5
		−3	5.0 ± 1.7	171 ± 17	33.8 ± 8.9
		−1	16.2 ± 3.6	488 ± 42	30.2 ± 4.4
		+1	23.3 ± 5.8	105 ± 12	4.5 ± 0.7
		+3	6.3 ± 2.1	194 ± 22	30.8 ± 8.1
		+5	4.8 ± 1.6	201 ± 21	41.6 ± 11.6
		C	3.8 ± 1.1	246 ± 18	65.2 ± 16.0
β-δ-SSB	1−50	−5	6.7 ± 2.8	242 ± 34	36.2 ± 12.0
		−3	10.7 ± 4.9	120 ± 19	11.2 ± 3.8
		−1	87.9 ± 33.8	518 ± 133	5.9 ± 0.8
		+1	23.3 ± 7.9	189 ± 28	8.1 ± 1.7
		+3	6.2 ± 3.7	267 ± 47	43.0 ± 21.2
		+5	2.6 ± 1.2	276 ± 33	104.8 ± 39.7
		C	3.0 ± 1.6	259 ± 40	87.5 ± 40.5

[a] K_M, k_{cat}, and specificity constant values represent the mean ± standard deviation of three experiments using six various concentrations (1, 2.5, 5, 10, 25, and 50 nM) of substrate [S]. The oligonucleotides with 8-oxoG on the labeled strand and a β-δ-SSB at positions +3 and +5 and the relative control DNA (containing 8-oxoG paired with cytosine) were incubated with 500 pg (2 nM) of hOGG1 whereas all other oligonucleotides were incubated with 1 ng (4 nM) of hOGG1.

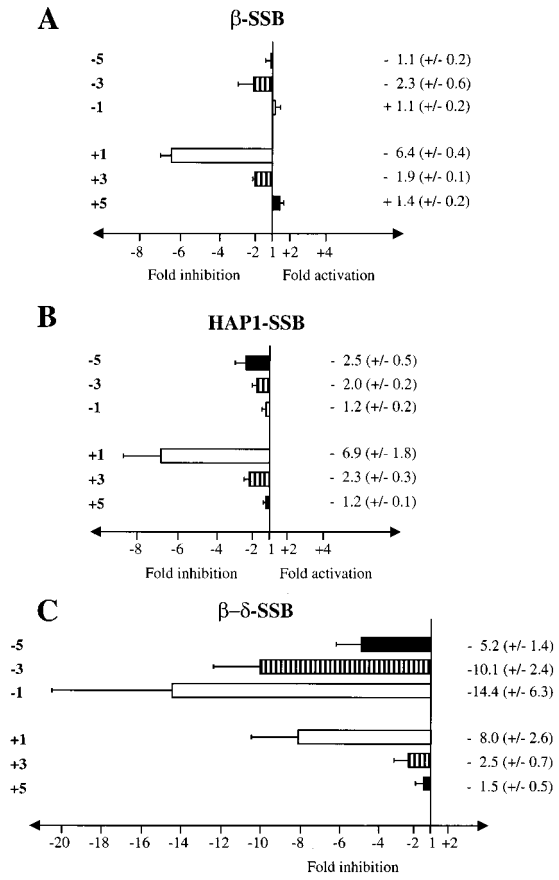

FIGURE 6: Effect of different types of single strand breaks, a β-SSB (A), an HAP1-SSB (B), or a β-δ-SSB (C), at the different positions -5 to $+5$ on the efficiency of excision of 8-oxoG by hOGG1 (0.25−0.5 ng), expressed as fold inhibition by comparison with the control oligonucleotide containing an 8-oxoG lesion. The error bars represent the standard deviation from five different experiments.

of k_{cat} show less variation compared with the value for the control oligonucleotide.

Binding of hOGG1 to 8-oxoG in the Vicinity of an Another Opposite Lesion. The binding of hOGG1 to oligonucleotides containing an AP site or a β-δ-SSB at positions -5 to $+5$ opposite to the site of 8-oxoG on the labeled complementary strand was investigated using gel shift assays (Figure 7), to assess whether the presence of the second lesion affects the recognition of 8-oxoG by hOGG1. As shown in Figure 7, a band shift representing binding of hOGG1 to the 8-oxoG-containing oligonucleotides was observed when an AP site is at any of the tested positions. The intensities of the shifted bands are similar to that with control DNA (lane C) with the exception of an AP site at position +1. The intensity of the shifted band is lower than that of the control DNA. Two shifted bands were observed with the oligonucleotide containing an AP site at position +5 opposite to the site of 8-oxoG. Whether the observation of these two bands represents binding of two molecules of hOGG1 per nucleotide is as yet not known.

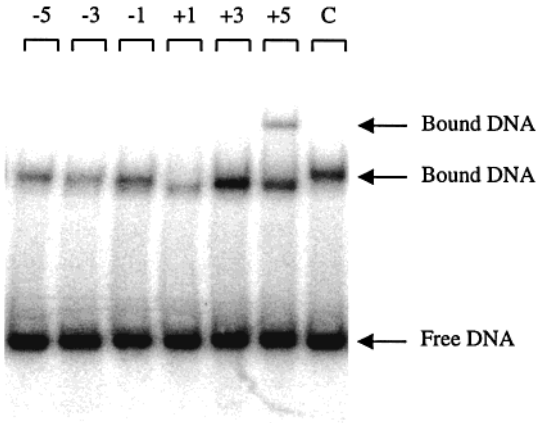

FIGURE 7: Gel profiles using EMSA showing the binding of hOGG1 protein (2.5 ng) to a ^{32}P-labeled oligonucleotide containing 8-oxoG as a single lesion (control probe, 0.5 nM, lane C) or in the presence of a neighboring AP site at positions -5 to $+5$ on the complementary nonlabeled strand.

DISCUSSION

The present study represents the first systematic investigation into assessing the influence of various types of neighboring DNA lesions on the excision of the highly mutagenic 8-oxoG by purified hOGG1 protein and XRS5 nuclear extracts, to gain insights into the initial step (excision step)

of the processing of clustered DNA damage. The presence of an opposite base damage (DHT, 8-oxoG) or uracil only slightly modifies, if at all, the efficiency of excision of 8-oxoG by XRS5 nuclear extracts and hOGG1. Any effect of two base lesions within a clustered DNA damage tends to be restricted to when they are closest neighbors, e.g., +1 separation. That a clustered damage containing a base lesion and an 8-oxoG on opposite DNA strands does not significantly inhibit the ability of BER enzymes to excise 8-oxoG was also seen with bacterial Nth and Fpg (*11*, *13*, *14*) and yeast OGG1 (*17*).

In contrast to these similarities, significant differences were seen between the levels of inhibition for excision of 8-oxoG in the presence of a neighboring AP site or reduced AP site with cell extracts when compared with that determined with hOGG1. Only when the AP site or reduced AP site is at position +1 is the extent of inhibition of excision of 8-oxoG similar with the extract and hOGG1. yOGG1 shows similar profiles of inhibition to those with hOGG1 (*17*). HOGG1 has a high affinity for AP sites when opposite a cytosine. In this study, the AP site is opposite adenine. Therefore, the AP site is probably not incised efficiently by hOGG1. Consequently, similar inhibition profiles are expected with AP and reduced AP sites, as seen from Figure 5. With nuclear extract, the rate of incision of an AP site or reduced AP site is greater than that for the glycosylase activity for excision of 8-oxoG (Figure 2). Further, the glycosylase activities of yOGG1 (*17*) and hOGG1 are greater than their associated, weak lyase activities. With XRS5 cell extracts, it is suggested that the inhibition of excision of 8-oxoG seen in the presence of an AP site or reduced AP site, extending over several base pairs separation, is mainly due to conversion of the AP site into a strand break by AP endonucleases in the extract. With XRS5 nuclear extracts, this latter process is rapid compared with that for excision of 8-oxoG, so that a SSB, which is formed rapidly at the AP site, is the lesion associated with the inhibitory effect on excision of 8-oxoG.

It was confirmed that SSBs, e.g., β-SSB, β-δ-SSB, and HAP1-SSB, do show a large inhibitory effect on excision of 8-oxoG by XRS5 nuclear extract and hOGG1, with the effect extending over several base pairs separation between the two lesions. Of the SSBs tested, a β-δ-SSB is more efficient in inhibiting the excision of 8-oxoG by nuclear extracts or by hOGG1 (Figures 4 and 6). That the inhibition extends over several base pairs separation may be very important, if formation of double strand breaks in cell is to be minimized during the processing of clustered DNA damage containing base modifications (data not shown) as discussed by Dianov et al. (*44*). These findings with hOGG1 are similar to those obtained previously with the yOGG1 (*17*). Since the strand break termini of the various SSB are different, the electrostatics within the clustered damage may influence the efficiency of the proteins to excise 8-oxoG.

That the AP lyase activity of hOGG1 is very weak was supported by the finding that HAP1 stimulates hOGG1 in its conversion of 8-oxoG into a SSB, presumably through rapid conversion of the intermediary AP site into a SSB (*45*, *46*). The catalytic constants determined for conversion of 8-oxoG, complementing cytosine in control oligonucleotide, into a SSB by hOGG1 (Table 2) are consistent with those reported for the AP lyase activity for removal of 8-oxoG by hOGG1 (*45*). In the kinetic studies reported here, the AP lyase activity of hOGG1 will be the rate-determining step when monitoring the formation of a SSB, since any AP sites present should be stable under the conditions used. Complications in the kinetic analysis reported by Hill et al. (*45*) probably reflect conversion of any residual AP sites present into SSB by the piperidine treatment.

The inhibition of excision of 8-oxoG by hOGG1 when an AP site or reduced AP site is at position +1 is reflected in reduced binding of hOGG1 to the oligonucleotide containing an AP site at position +1. From structural studies of binding of hOGG1 to DNA containing 8-oxoG, it was proposed (*47*) that specific amino acid residues of hOGG1 interact with the partner cytosine of 8-oxoG on the complementary strand through insertion into the space on the 5′ and 3′ sides of the cytosine. This insertion causes a kink in the DNA through unstacking of the neighboring bases with the cytosine. Interactions of the protein with bases >1 bp away from the cytosine were not observed. Since the reduced binding/excision of hOGG1 to 8-oxoG is seen when an AP is at position +1, the interaction of the amino acid residues with the cytosine on its 3′ side may have a larger influence on the interaction of hOGG1 with 8-oxoG, together with any distortions in the oligonucleotides caused by an AP site at site +1.

The observation of two shifted bands when an AP site is at position +5 to the 8-oxoG present on the complementary strand may represent binding of two molecules of hOGG1 per oligonucleotide. From structural studies (*47*) and footprinting (*48*), bound hOGG1 protects, respectively, 2 and 3 nucleotides 3′ and 5′ of an AP site, which is refractory to incision, and the complementary base to the lesion. With 8-oxoG, bound OGG1 protects 2−3 nucleotides 3′ and 5′ of the 8-oxoG and its partner cytosine. Therefore, it is possible that one molecule of OGG1 could bind to 8-oxoG and one to the AP site on the complementary strand, provided that the two lesions are separated by at least 4 bp.

In summary, the effect of a neighboring lesion on the efficiency of excision of 8-oxoG within a clustered DNA damage is similar for both yOGG1 (*17*) and hOGG1. With purified enzymes a neighboring AP site does not significantly inhibit excision of 8-oxoG whereas, with extracts, the excision of 8-oxoG is significantly inhibited by the presence of an AP site due to its conversion into a SSB by AP endonucleases. Therefore, AP endonucleases may play a role in orchestrating the way in which clustered DNA damage containing base lesions is processed in cells.

ACKNOWLEDGMENT

We are grateful to Profs. Rick Wood (Imperial Cancer Research Fund) and Ian Hickson (Institute of Molecular Medicine) for providing the purified Nth and HAP1 proteins, respectively. S.B. thanks the Association pour la Reserche sur le Cancer (ARC-5432).

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BI0112356