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APE1-dependent repair of DNA single-strand breaks containing 3′-end 8-oxoguanine

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ABSTRACT

DNA single-strand breaks containing 3′-8-oxoguanine (3′-8-oxoG) ends can arise as a consequence of ionizing radiation and as a result of DNA polymerase infidelity by misincorporation of 8-oxodGMP. In this study we examined the mechanism of repair of 3′-8-oxoG within a single-strand break using purified base excision repair enzymes and human whole cell extracts. We find that 3′-8-oxoG inhibits ligation by DNA ligase IIIα or DNA ligase I, inhibits extension by DNA polymerase β and that the lesion is resistant to excision by DNA glycosylases involved in the repair of oxidative lesions in human cells. However, we find that purified human AP-endonuclease 1 (APE1) is able to remove 3′-8-oxoG lesions. By fractionation of human whole cell extracts and immunoprecipitation of fractions containing 3′-8-oxoG excision activity, we further demonstrate that APE1 is the major activity involved in the repair of 3′-8-oxoG lesions in human cells and finally we reconstituted repair of the 3′-8-oxoG-containing oligonucleotide duplex with purified human enzymes including APE1, DNA polymerase β and DNA ligase IIIα.

INTRODUCTION

Ionizing radiation produces DNA lesions largely through the generation of reactive oxygen species (ROS) that attack DNA (1). The same types of ROS are generated endogenously as by-products of oxidative metabolism and during the inflammatory response in non-irradiated cells. ROS can induce oxidative DNA damage, such as 8-oxoguanine (8-oxoG), directly or can oxidize nucleotide triphosphates that can be later incorporated into DNA (2). Furthermore, several events may lead to the formation of DNA lesions containing 3′-terminal 8-oxoG residues. For example during base excision repair (BER), misincorporation of 8-oxodGMP by DNA polymerase β lacking proofreading activity may generate a 3′-terminal 8-oxoG (3,4). If this 3′-terminal 8-oxoG does affect ligation of the neighbouring phosphodiester bond, then it will result in persistent DNA containing single-strand breaks (SSBs) with 3′-terminal 8-oxoG ends. Similar lesions may also arise as a result of so-called 'clustered lesions', since ionizing radiation energy is deposited in small volumes of nanometre dimensions resulting in ROS being produced at high local concentrations (5). Consequently, the formation of multiply damaged sites in DNA may include 3′-8-oxoG lesions immediately next to a SSB.

Normally 8-oxoG, as with many other base lesions in DNA, is efficiently repaired by the BER pathway (6,7). However, little is known about the repair of base lesions located in close proximity to DNA strand breaks, although early findings indicated that repair enzymes could inefficiently process such lesions. For example, uracil-DNA glycosylase inefficiently removes uracil residing at the 3′-end of oligonucleotides (8) and DNA polymerase β, the major base excision repair polymerase, very inefficiently extends a primer containing 3′-terminal 8-oxoG (3,9). Therefore, the mechanism for the repair of 3′-terminal 8-oxoG lesions is unclear although there are several repair pathways that have the potential to process these 3′-blocking lesions, including direct excision of the 3′-terminal 8-oxoG by DNA glycosylase activity or a specific 3′-end processing activity that would excise the modified nucleotide. This would be followed by the repair of the intermediate product arising by proteins of the BER pathway.

In this study, we use oligonucleotide duplexes containing a site-specific lesion and human whole cell extracts to address the mechanism of repair of 3′-8-oxoG lesions within a DNA SSB.

MATERIALS AND METHODS

Materials

Synthetic oligodeoxyribonucleotides were purchased from MWG-Biotech and gel purified on a 20% polyacrylamide gel. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Human AP-endonuclease 1 (APE1), Pol β and DNA ligase IIIα were purified as described previously (10). His-tagged OGG1 and DNA ligase IIIα were purified on a

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Ni-column as recommended by the manufacturer. NEIL1 and NEIL2 were kindly provided by D. Zharkov and DNA ligase I was a gift from A. Tomkinson. Antibodies against human APE1 were raised in rabbit and affinity purified as described (11).

Substrate labelling

Oligonucleotides were 5′-end labelled with [γ-^{32}P]ATP using T4-polynucleotide kinase and unincorporated label removed on a Sephadex G-25 spin column. To prepare the 3′-8-oxoG substrate, the oligonucleotides 5′-CCTGCAGGTCGACTCTAGA^{8OXO}G-3′ and 5′-GCCGGCCGATCAAGCTTATTGGGT-3′ were annealed to a 2-fold excess of the oligonucleotide 5′-ACCCAATAAGCTTGATCGGCCGGCCTCTAGAGTCGACCTGCAGG-3′ (8-oxoG/C) or 5′-ACCCAATAAGCTTGATCGGCCGGCATCTAGAGTCGACCTGCAGG-3′ (8-oxoG/A) at 90°C for 3–5 min followed by slow cooling to room temperature. To prepare the control containing substrate, the oligonucleotide 5′-CCTGCAGGTCGACTCTAGAG-3′ replaced the 8-oxoG containing oligonucleotide.

Fractionation of cell extracts

HeLa cell pellets were purchased from Paragon, USA. Whole cell extracts (WCEs) were prepared by the method of Manley *et al*. (12) dialysed overnight against buffer containing 25 mM HEPES–KOH, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol and 2 mM DTT and aliquots frozen at −80°C for the repair assays. An aliquot of this extract (100 mg protein) was then fractionated by Phosphocellulose chromatography using a step elution of 150 mM KCl (PC-FI) and 1 M KCl (PC-FII) as previously described (13,14). Proteins (10 mg) from the PC-FII fraction were further separated by gel filtration on a Superdex-75 column (Amersham) in a buffer containing 50 mM HEPES (pH 7.9), 150 mM KCl, 1 mM EDTA, 1 mM DTT and 1 mM phenylmethylsulphonyl fluoride. 0.5 ml fractions were collected and tested for the ability to remove 3′-8-oxoG as described below.

Repair assays using purified enzymes

Assays using purified proteins contained 250 fmol oligonucleotide per reaction in 20 µl Reaction buffer containing 50 mM HEPES–KOH, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1.5 mM DTT, 8.5% glycerol and 100 µg/ml BSA. Primer extension and *in vitro* reconstitution reactions also included 20 µM of each dCTP, dATP, dGTP and TTP. All reactions were incubated for the time indicated at 37°C and 20 µl formamide loading dye (95% formamide, 0.02% xylene cyanole, 0.02% bromophenol blue) added and the samples heated to 95°C for 5 min. Products were subsequently analysed by 20% denaturing PAGE and gels exposed to intensifying screens at 4°C prior to analysis by phosphorimaging.

Excision and repair of 3′-8-oxoG by cell extracts

Excision assays contained 250 fmol oligonucleotide per reaction in 20 µl reaction buffer containing 50 mM HEPES–KOH, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1.5 mM DTT, 8.5% glycerol, 2 mM ATP, 25 mM phosphocreatine (diTris salt, Sigma), 2.5 µg creatine phosphokinase (type I, Sigma), 0.25 mM NAD^+ and 1 µg of carrier DNA (single stranded 30mer oligonucleotide). Repair reactions also included 20 µM of each dNTPs. Reactions were incubated for the time indicated at 37°C, 20 µl formamide loading dye added and samples heated to 95°C for 5 min. Products were subsequently analysed by 20% denaturing PAGE and gels exposed to intensifying screens at 4°C prior to analysis by phosphorimaging.

Immunodepletion of APE1

Protein A Sepharose CL-4B was allowed to swell for 1 h in phosphate-buffered saline (PBS) and following washes with PBS a 50%-suspension was prepared. To 100 µl suspension was added 5 µl APE1 antibodies or 5 µl pre-immune serum and incubation was carried out for 2 h at 4°C with gentle shaking. The APE1-Sepharose was washed five times with 0.3 ml PBS and resuspended in 100 µl PBS. Fractions purified from Phosphocellulose and gel filtration chromatography (20 µl) were mixed with 20 µl APE1-Sepharose and incubated for 2 h at 4°C with gentle shaking. The mixture was filtered through Spin-X columns at 4°C and aliquots taken and SDS-PAGE loading dye (25 mM Tris–HCl, pH 6.8, 2.5% mercaptoethanol, 1% SDS, 5% glycerol, 1 mM EDTA, 0.15 mg/ml bromophenol blue) added. The samples were heated to 90°C for 3 min prior to loading on a 10% SDS–polyacrylamide gel followed by transfer to a PVDF membrane and immunoblot analysis with the indicated antibodies. Aliquots were also taken for 3′-8-oxoG repair assays as described above.

RESULTS

3′-8-oxoG residues inhibit ligation by DNA ligases and processing by DNA polymerase β or DNA glycosylases

The easiest way to process 3′-8-oxoG is to remove it by a DNA glycosylase activity. To test for DNA glycosylase activities towards 3′-8-oxoG residues, the 3′-8-oxoG containing oligonucleotide was 5′-labelled prior to construction of the nick containing duplex oligonucleotide (Figure 1A). This substrate

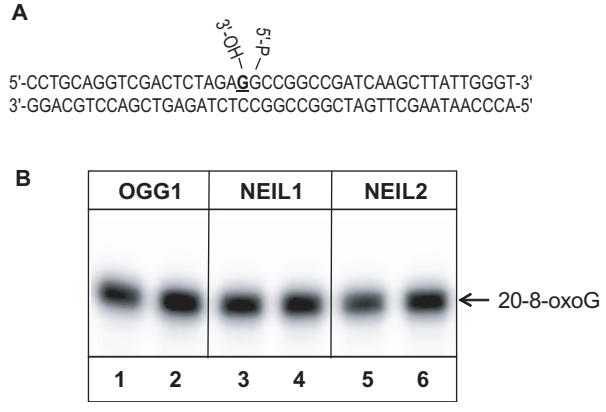

Figure 1. The human DNA glycosylases OGG1, NEIL1 and NEIL2 are unable to excise 3′-8-oxoG lesions. (**A**) Schematic representation of the 3′-8-oxoG containing oligonucleotide substrate within a SSB. Underlined bold **G** stands for 8-oxodG. (**B**) The 20mer 3′-8-oxoG containing oligonucleotide substrate (20-8oxoG, 0.25 pmol) was incubated with OGG1, NEIL1 or NEIL2 (2.6, 2.6 and 2.3 pmol, respectively) for 20 min at 37°C prior to the addition of formamide loading dye with (lanes 1, 3 and 5) or without (lanes 2, 4 and 6) 100 mM NaOH. Samples were heated to 95°C for 5 min prior to analysis by 20% denaturing PAGE and phosphorimaging.

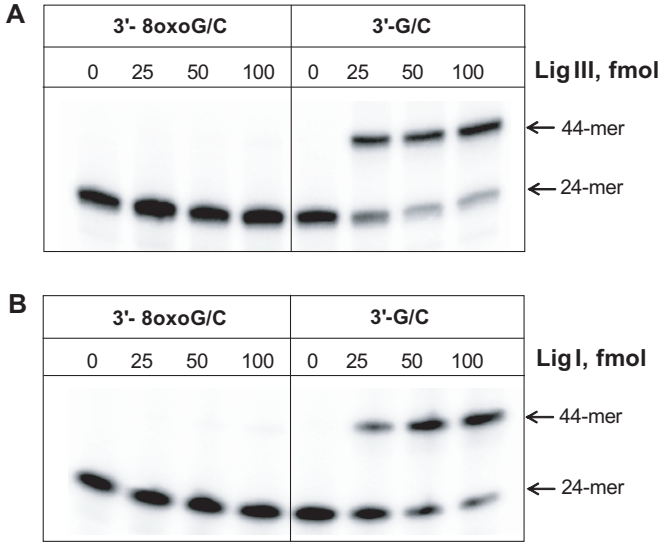

Figure 2. An oligonucleotide containing 3′-8-oxoG within a SSB cannot be ligated by DNA ligase IIIα or DNA ligase I. 250 fmol of the 3′-8-oxoG containing (left panel) or a control duplex oligonucleotide substrate (right panel) were incubated with DNA ligase IIIα (**A**) or DNA ligase I (**B**) for 20 min at 37°C prior to the addition of formamide loading dye. An aliquot was analysed by 20% denaturing PAGE and phosphorimaging.

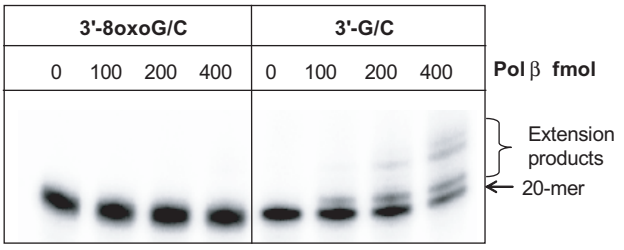

Figure 3. An oligonucleotide containing 3′-8-oxoG ends is resistant to primer extension by DNA polymerase β. 250 fmol of the 3′-8-oxoG containing (left panel) and a control duplex oligonucleotide substrate (right panel) were incubated with DNA polymerase β (0–400 fmol) for 20 min at 37°C prior to the addition of formamide loading dye. An aliquot was analysed by 20% denaturing PAGE and phosphorimaging.

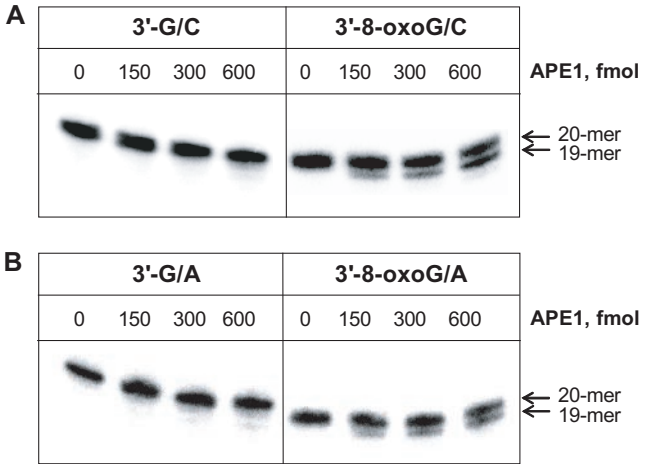

Figure 4. APE1 excises 3′-8-oxoG lesions present within a SSB. 250 fmol of the duplex oligonucleotide substrate containing 3′-8-oxoG opposite to C (3′-8-oxoG/C; **A**, right panel) or A (3′-8-oxoG/A; **B**, right panel) and a control duplex containing 3′-G/C base pair (A, left panel) or 3′-G/A mismatch (B, left panel) were incubated with APE1 (0–600 fmol) for 20 min at 37°C prior to the addition of formamide loading dye. An aliquot was analysed by 20% denaturing PAGE and phosphorimaging.

was subsequently incubated with a 10-fold molar excess of either human 8-oxoguanine DNA glycosylase (OGG1) or human endonuclease VIII-like proteins (NEIL1, NEIL2). Although the activity of all enzymes had been tested with the corresponding substrates (data not shown), no excision activity against 3′-8-oxoG lesions could be observed (Figure 1B). Furthermore, on addition of sodium hydroxide to cleave any abasic sites generated by DNA glycosylase activity no cleavage products were observed indicating that, at the concentrations used, DNA glycosylase activities of OGG1, NEIL1 or NEIL2 are not active against 3′-8-oxoG residues.

Another opportunity to process 3′-8-oxoG would be to convert it to a conventional 8-oxoG by sealing the SSB and then repair it through the BER pathway. To investigate this possibility, a radiolabelled duplex oligonucleotide substrate containing a 5′-phosphorylated nick with 3′-8-oxoG ends was constructed (Figure 1A). The ability of DNA ligase IIIα and DNA ligase I to ligate this substrate was then compared to the corresponding control oligonucleotide containing 3′-guanine. The control duplex oligonucleotide was gradually ligated by increasing concentrations of DNA ligase to the corresponding 44mer oligonucleotide (Figure 2A and 2B, right panel). However the 3′-8-oxoG containing oligonucleotide is non-ligatable, even at the highest amount of DNA ligase used (Figure 2A and 2B, left panel).

Another possibility for removing 3′-8-oxoG lesions would be to extend the 3′-end by DNA polymerase synthesis and afterwards repair 8-oxoG through the BER pathway. To investigate such a possibility, a substrate containing the 20mer 5′-labelled 3′-8oxoG lesion annealed to the 44mer complementary oligonucleotide was generated to analyse the ability of Pol β (the major BER polymerase) to extend the 3′-8-oxoG containing primer. With a control oligonucleotide substrate, primer extension is achieved by increasing concentrations of Pol β (Figure 3, right panel). However, under the same conditions the 3′-8-oxoG containing substrate is resistant to primer extension synthesis by Pol β (Figure 3, left panel). These experiments show that 3′-8-oxoG residues are non-ligatable, resistant to DNA glycosylase activities and are not extended by Pol β, and indicate a specific repair enzyme for the removal of 3′-8-oxoG residues. It has been previously shown that APE1 has 3′-exonuclease activity that is involved in the removal of 3′-end mismatched nucleotides (15) and therefore we tested the possibility of purified APE1 being capable of excising 3′-8-oxoG lesions. With the use of a control nick containing duplex oligonucleotide (3′-G/C) or mismatch containing duplex (G/A), no significant exonuclease activity of APE1 was observed in our reaction conditions (Figure 4A and B; left panel). In comparison, using a 3′-8-oxoG nick containing duplex oligonucleotide substrate with 8-oxoguanine opposite cytosine (3′-8oxoG/C) or adenine (3′-8oxoG/A), APE1 processed the 3′-end specifically removing the 8-oxoG lesion only to generate a 19mer product (Figure 4A and B; right panel) and demonstrates the 3′-8-oxoG specific end processing activity of APE1.

Fractionation of 3′-8oxoG excision activity from human WCE

Although we showed that purified APE1 is able to cleave 3′-8-oxoG residues, we tested human WCE for the ability to repair this lesion and subsequently isolated the major activity against 3′-8-oxoG. Using HeLa WCE, we were able to demonstrate the repair of 3′-8-oxoG residues when present within a nick, although with low efficiency. Incubation with WCE converted the 20mer 3′-8-oxoG containing labelled fragment of the oligonucleotide substrate, shown in Figure 1A, to the full-length repaired 44mer product, indicating the 3′-8-oxoG excision activity in WCE (Figure 5A). We proceeded to further characterize the 3′-8-oxoG activity from human WCE using Phosphocellulose chromatography, eluting proteins off into two fractions using low (0.15 M KCl, PC-FI) and high salt (1 M KCl, PC-FII) elution (13,14). These fractions were tested for their ability to remove 3′-8-oxoG lesions and we find that the majority of the 3′-8-oxoG activity observed in human WCE is retained in the high salt elution fraction PC-FII only (Figure 5B; fourth panel), which has also been found to contain the majority of BER proteins, including APE1 (7). No other residual activity for the removal of 3′-8-oxoG was observed in the low salt fraction PC-FI (Figure 5B; third panel). The proteins within fraction PC-FII, which contained the major 3′-8-oxoG excision activity, were further separated according to their size by gel filtration chromatography. The fractions were subsequently tested for their ability to remove 3′-8-oxoG lesions and it was shown that the activity was present predominantly within fractions 19–23, where proteins with a molecular weight of ~40–60 kDa are eluted (Figure 5C). Western blot analysis of these fractions revealed that elution of APE1 occurred within fractions 19–23 (Figure 5D) so we conclude that the 3′-8-oxoG cleavage activity co-purifies with APE1 protein.

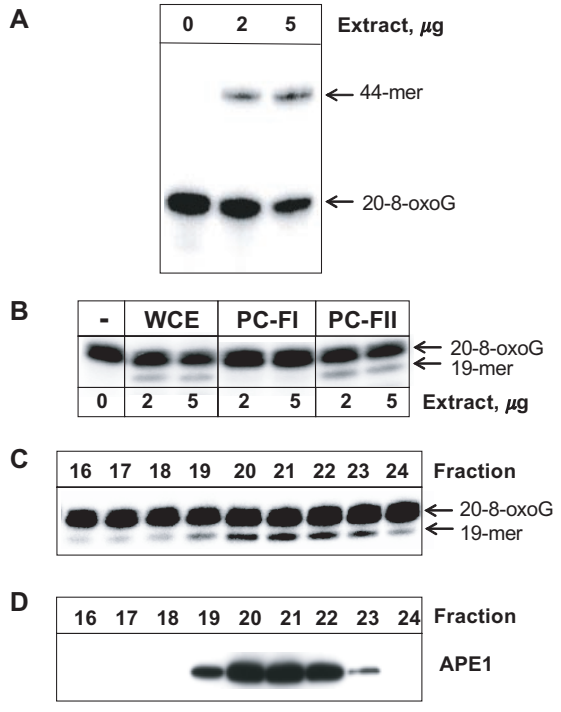

Figure 5. Partial purification of 3′-8-oxoG end processing activity from human cell extracts using Phosphocellulose and gel filtration chromatography. (**A**) Repair of the duplex oligonucleotide substrate containing 3′-8-oxoG by HeLa WCE. (**B**) HeLa WCE was loaded onto a Phosphocellulose column and fractions were step eluted using 0.15 M (PC-FI) and 1 M (PC-FII) KCl. 250 fmol of the 3′-8-oxoG nick containing duplex oligonucleotide substrate was incubated with WCE or Phosphocellulose fractions (2 and 5 μg) in the absence of dNTPs for 20 min at 37°C. Formamide loading dye was added and an aliquot analysed by 20% denaturing PAGE and phosphorimaging. (**C**) Proteins from PC-FII were further separated by gel filtration on a Superdex-75 column and the fractions obtained were analysed for 3′-8-oxoG activity using 250 fmol of the 3′-8-oxoG duplex oligonucleotide substrate by 20% denaturing PAGE and phosphorimaging. (**D**) Aliquots of the fractions were also analysed by SDS–PAGE and western blotting using APE1 antibodies.

Co-precipitation of APE1 and 3′-8-oxoG activity

Gel filtration fractions containing the peak of 3′-8-oxoG excision activity (fractions 20 and 21) were chosen to immunodeplete APE1 using antibodies raised against APE1. Analysis by western blot reveals that, although the levels of APE1 are slightly reduced by mock immunodepletion compared to the original fractions, using APE1 antibodies the fractions were completely depleted of the APE1 protein (Figure 6A). The original fractions, alongside the mock immunodepleted and immunodepleted fractions, were subsequently tested for their ability to remove 3′-8-oxoG lesions. The mock immunodepletion protocol does not affect 3′-8-oxoG cleavage activity in fractions 20 and 21 (Figure 6B, lanes 4 and 5) compared to the original fractions (Figure 6B, lanes 2 and 3). However, using antibodies specific to APE1, the activity against 3′-8-oxoG is completely ablated (Figure 6B, lanes 6 and 7). Taken together, these results indicate that APE1 is the major enzyme processing 3′-8-oxoG lesions in human cell extracts.

Finally, we reconstituted repair of the 3′-8-oxoG containing duplex oligonucleotide with purified human enzymes including APE1, Pol β and DNA ligase IIIα (Figure 7). Addition of APE1 converted the 20mer fragment containing 3′-8-oxoG into a 19mer (Figure 7, lane 2), which is observed as the 20mer fragment after filling the gap by Pol β (Figure 7, lane 3) and

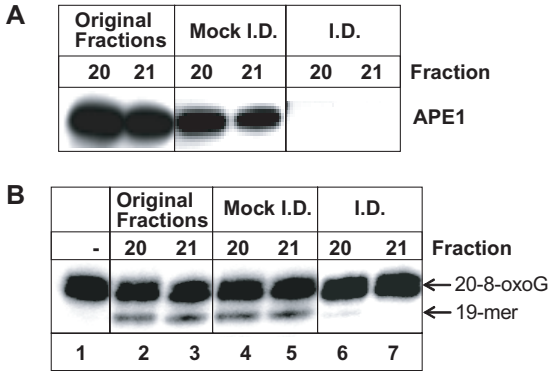

Figure 6. Immunoprecipitation of APE1 from gel filtration fractions containing 3′-8-oxoG end processing activity. (**A**) Fractions from gel filtration chromatography containing 3′-8-oxoG excision activity (fractions 20 and 21) were mock-immunodepleted and immunodepleted of APE1 using APE1 specific antibodies. Samples were analysed by SDS–PAGE and western blotting using antibodies against APE1. (**B**) The original fractions, mock immunodepleted and immunodepleted fractions 20 and 21 were tested for 3′-8-oxoG activity using 250 fmol of the 3′-8-oxoG containing duplex oligonucleotide. Samples were incubated for 10 min at 37°C prior to the addition of formamide loading dye and analysis by 20% denaturing PAGE and phosphorimaging.

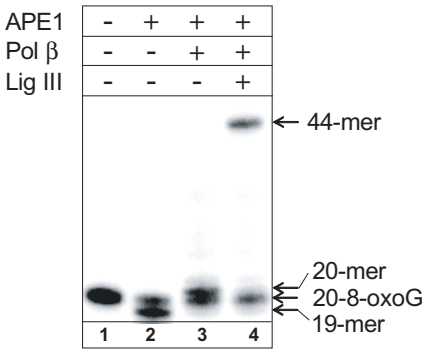

Figure 7. Reconstitution of the repair pathway for DNA SSBs containing 3′-8-oxoG. 250 fmol of the 3′-8-oxoG containing duplex oligonucleotide substrate was incubated with 2 pmol of APE1, 100 fmol of Pol β and 100 fmol of DNA ligase IIIα at the indicated combinations for 20 min at 37°C prior to the addition of formamide loading dye. An aliquot was analysed by 20% denaturing PAGE and phosphorimaging.

finally DNA ligase seals the DNA ends generating a 44mer full-length repair product (Figure 7, lane 4).

DISCUSSION

Ionizing radiation and the misincorporation of 8-oxodGMP by DNA polymerases can induce SSBs with terminal 3′-8-oxoG ends. However, several different repair mechanisms exist that may potentially repair this lesion. Excision of 3′-8-oxoG ends by a DNA glycosylase is one potential repair pathway however, previous reports on the removal of 3′-end damaged bases are conflicting as 3′-end uracil residues are resistant to cleavage by uracil-DNA glycosylase (8) whereas it has been suggested that human endonuclease III would be able to excise 3′-terminal dihydrouracil residues (16). OGG1 was discovered as the major enzyme for the removal of 8-oxoG:C pairs in DNA (17,18) although the emergence of NEIL proteins that contain a wide substrate specificity (19–21) may serve as a backup activity to OGG1, even though NEIL1 has a relatively weak activity towards 8-oxoG lesions in comparison to OGG1. However, we found that the 3′-8-oxoG lesion is resistant to excision by OGG1, NEIL1 and NEIL2 even when a 10-fold molar excess of DNA glycosylases has been used.

Repair of SSBs containing 3′-8-oxoG lesions could proceed by direct ligation by a DNA ligase, so that the 8-oxoG lesion can then be processed by the BER pathway. Relatively little is known about the ligation of SSBs containing 3′-8-oxoG using human DNA ligases, although it has been previously reported that T4 DNA ligase can ligate oligonucleotide substrates containing 3′-8-oxoG ends (22). However, our studies using either human DNA ligase I or IIIα demonstrated that the 3′-8-oxoG containing strand break is non-ligatable under physiological conditions.

Another possible solution for the repair of 3′-8-oxoG residues would involve DNA polymerase strand displacement synthesis so that the 3′-end would be moved downstream to the lesion and 8-oxoG can be subsequently repaired by the BER pathway. Previous reports suggest that phage T7 DNA polymerase and *E.coli* polymerase I or II are able to undergo primer extension from 3′-8-oxoG lesions, although the reactions are generally inefficient (9,22). DNA polymerase α and β have also been shown to extend from 3′-8-oxoG residues, although with low efficiency (3) and in agreement with this study our results show that Pol β is unable to efficiently extend beyond 3′-8-oxoG lesions.

We subsequently found that APE1 protein can excise 3′-8-oxoG lesions and furthermore, APE1 activity is the predominant mechanism for excision of 3′-8-oxoG lesions in human cell extracts. This would then create a one nucleotide gap substrate for gap filling and ligation by a DNA polymerase and a DNA ligase, respectively. Correspondingly, we were able to reconstitute the entire pathway with purified human enzymes including APE1, Pol β and DNA ligase IIIα. APE1 is known to interact with XRCC1 (23) and Pol β (24) during BER and subsequently these interactions may be important during the repair of 3′-8-oxoG lesions initiated by APE1, for the recruitment of Pol β and XRCC1-DNA ligase IIIα complex that will restore DNA integrity.

Taken together, our data demonstrate that APE1 is the major activity involved in the repair of SSBs containing 3′-8-oxoG in human cell extracts. We have previously demonstrated that APE1 is also the major activity in human cell extracts against 3′-phosphoglycolate (14). Furthermore, APE1 has been shown to contain 3′-exonuclease activity for mismatched DNA bases [for review see (25)]. These observations support the view that APE1 may play a central role in the processing of SSBs containing blocked 3′-ends. Our data also predict that APE1 may excise other 3′-end modified nucleotides that are resistant to cleavage by DNA glycosylases. Consistent with our results, it was recently demonstrated that yeast cell-free extracts, as well as purified Apn1, can excise 3′-8-oxoG and that deletion of both *OGG1* coding for 8-oxoG-DNA yeast glycosylase and *APN1* coding for the major yeast AP endonuclease causes a 46-fold synergistic increase in spontaneous mutation rate (M. Saparbaev, personal communication).

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Conflict of interest statement. None declared.

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