Comparative repair of the endogenous lesions 8-oxo-7,8-dihydroguanine (8-oxoG), uracil and abasic site by mammalian cell extracts: 8-oxoG is poorly repaired by human cell extracts

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Introduction
DNA damage of endogenous origin may significantly contribute to human cancer. In particular the oxidized base 8-oxo-7,8-dihydroguanine (8-oxoG), the product of deamination of cytosine, uracil (U) and the sites of base loss (AP sites) are among the most frequent mutagenic lesions formed in the human genome under physiological conditions. According to a recent survey (T. Lindahl, quoted in ref. 1), ~1000 8-oxoG, ~400 U and ~9000 AP site residues are generated daily per human genome. To cope with this huge load of damage, cells are equipped with different repair mechanisms. One important repair mechanism dealing with endogenous damage is DNA base excision repair (BER) (2,3). Two distinct BER pathways have been demonstrated in mammalian cells: a single nucleotide insertion pathway, catalyzed by DNA polymerase β (4,5) and a proliferating cell nuclear antigen (PCNA)-dependent pathway, involving a resynthesis patch of 2–10 nucleotides (6–8). In human cells, BER of 8-oxoG is initiated by a bifunctional glycosylase/AP lyase activity termed human oxoguanine glycosylase 1 (hOGG1) the coding sequence of which has been cloned (9,10). Repair of U is initiated by a major U DNA glycosylase encoded by the UNG gene (11). The UNG protein represents >98% of the total U-DNA glycosylase activity in human cells and has no associated AP lyase activity (11). Removal of U therefore leads to generation of a natural AP site that is incised by the major human AP endonuclease variously termed HAP1/APE/Ref-1 (3).

We have investigated here the repair capacities of human cell extracts for 8-oxoG, U and natural AP site. We report that 8-oxoG is poorly repaired in comparison with the other two lesions.

Materials and methods

Cell culture
Normal human fibroblasts GM 5757 were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ) and cultured as recommended.

DNA plasmid substrates
The procedures for preparation of plasmids carrying a single lesion have been described previously (6,12, Figure 1). Briefly, pGEM3Zf (+) single-stranded DNA was annealed with an oligonucleotide (22 bp, GATCCTCTA-GAGTCGACCTGCA) carrying a single 8-oxoG in position 12 [TIBMOB-BIOL, Genova, Italy; (pGEM 8-oxoG)] or a single U in position 13 (pGEM U), thus generating a single 8-oxoG/cytosine base pair or a single U/adenine base pair. Control pGEM T plasmids were prepared with an oligonucleotide carrying the normal bases guanine and thymine. Closed circular double-stranded DNA was obtained by incubating with T4 DNA polymerase, single-strand binding protein and T4 DNA ligase. The plasmid carrying a single natural AP site [pGEM U (ura)] was generated by incubation of pGEM U with Escherichia coli U DNA glycosylase (1 ng protein/50 ng DNA, 37°C for 45 min). The plasmid carrying a single β-elimination-generated AP site incision [pGEM U (ura) (nth)] was obtained by incubating pGEM U (ura) with E.coli endonuclease III (nth) protein (1 ng protein/30 ng DNA, 37°C for 10 min). Characterization of pGEM U and pGEM U (ura) plasmid substrates has been described (6). Plasmids were purified by equilibrium density gradient centrifugation with cesium chloride (12). The relative amount of nicked circular forms (Form II) did not exceed 10% of the total DNA molecules.

Extracts
They were prepared by the method of Tanaka et al. (13) as described in Biades et al. (8). Briefly, exponentially growing cells were harvested from four 175 cm2 flasks, washed three times with phosphate-buffered saline and resuspended in buffer I (10 mM Tris–HCl pH 7.8, 200 mM KCl), at a concentration of 5×10^7 cells/ml. After addition of an equal volume of buffer II (10 mM Tris–HCl pH 7.8, 200 mM KCl, 2 mM EDTA, 40% glycerol, 0.2% Nonidet P-40, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 5 µg/ml leupeptin, 1 µg/ml pepstatin), the cell suspension was stirred for 1 h at 4°C and centrifuged at 16,000 g for 10 min. The supernatant was dispensed into aliquots and stored in liquid nitrogen.

In vitro BER assay
The in vitro BER assay was employed as described (6,12). Briefly, 300 ng of plasmid substrate were incubated with 30 µg of extract protein for the indicated times at 30°C. [32P]dUTP was the label of choice when the single nucleotide insertion pathway was under investigation, on pGEM U or pGEM U (ura), as the single U is located opposite dAMP. [32P]dGTP was the label of choice when the single nucleotide insertion pathway was under investigation on pGEM 8-oxoG. Finally, [32P]dCTP was the label of choice when the PCNA-dependent pathway was under investigation with any substrate because, within the HinII–PstI fragment located 3’ to the lesion, cytosine is the most represented base (three out of eight bases) (Figure 1). After the repair reaction, the DNA reaction product was purified and treated with the restriction endonucleases XhoI–HinII or HinII–PstI (20 units each) whether the single nucleotide insertion or the PCNA-dependent pathway were under investigation, respectively. The resulting fragments (8mer in both cases) were resolved by polyacrylamide gel electrophoresis in the presence of 7 M urea at 30 mA.
The capacity of normal human fibroblasts to repair a single 8-oxoG, U or AP site by the one nucleotide insertion pathway is shown in Figure 2A. pGEM T (lane 1), pGEM 8-oxoG (lanes 2–4), pGEM U (lanes 5–7) and pGEM U (ura) (lane 8) plasmids were incubated with 30 µg of protein of GM 5757 extract for 1 (lanes 2, 5 and 8), 3 (lanes 3 and 6) and 6 h (lanes 1, 4 and 7). Quantification of data is shown in Figure 2B. At 1 h incubation time, the repair replication stimulated by 8-oxoG was undetectable while the single AP site was repaired on average 10-fold better than the single U (350 versus 35 c.p.m.). Repair of a single AP site reaches completion by this time (14,17). At 3 and 6 h incubation time, the ratio of repair incorporation between U and 8-oxoG substrates was 5.7- and 4.5-fold, respectively (mean: 5.1-fold). Hence, a single natural AP site was repaired on average 10×5 = 50-fold better than a single 8-oxoG. No background incorporation could be observed on pGEM T control plasmids incubated for 6 h under the same conditions used for pGEM U and pGEM U (ura) plasmids (lane 1).

The poor repair of 8-oxoG observed in human cell extracts was also a feature of AA8 Chinese hamster cell extracts. Yet, in this case, less marked differences were observed for the three lesions as a single 8-oxoG was repaired 3-fold less efficiently than a single U that, in turn, was repaired 3.1-fold less efficiently than a single AP site (data not shown).

The capacity of GM 5757 human extracts to perform the PCNA-dependent BER pathway on 8-oxoG, U and natural AP site is shown in Figure 3. The experiment was performed using [32P]dCTP as labeled nucleotide and a restriction endonuclease carrying the single 8-oxoG or the single U with the complementary sequence. The reactions were carried out under the same conditions used for the in vitro BER assay. Thereafter, the DNA substrates were purified, divided into three aliquots and enzymatically hydrolyzed (15). 8-oxoG and dU were quantitated by HPLC coupled to an electrochemical or an ultraviolet detector (absorbance wavelength = 254 nm), respectively.

Results

Characterization of plasmid substrate containing a single 8-oxoG

The capacity of GM 5757 cells to perform either the single nucleotide insertion or the PCNA-dependent pathways on 8-oxoG, U and natural AP site, was evaluated using plasmid substrates carrying single lesions at a defined position (6). Figure 1 shows a schematic representation of pGEM 8-oxoG, pGEM U, pGEM U (ura) and pGEM T (control) plasmid substrates with relevant restriction sites XbaI, HindII and PstI. pGEM 8-oxoG was characterized as following. First, the composition of the 22mer carrying a single 8-oxoG used for plasmid preparation was verified by mass spectral analysis, showing a difference of 15 Da between its molecular weight and that of the control oligo (data not shown). Second, the 8-oxoG oligomer was annealed to the complementary oligonucleotide and the duplex enzymatically hydrolyzed (15). 8-oxoG was quantitated by HPLC coupled to an electrochemical detector (16). Table I shows the results of three independent analyses performed by injecting 0.8 µg of hydrolyzed duplex. An average ratio of 11.53 (against an expected ratio of 11) residues of dG every 8-oxoG was found, thus showing the correct composition of the oligomer. Third, pGEM 8-oxoG plasmid was specifically sensitive to the AP lyase activity of fpg protein, with complete conversion from closed circular to nicked circular forms (data not shown).

Comparative BER of 8-oxoG, U and natural AP site

The capacity of normal human fibroblasts to repair a single 8-oxoG, U or AP site by the one nucleotide insertion pathway is shown in Figure 2A. pGEM T (lane 1), pGEM 8-oxoG (lanes 2–4), pGEM U (lanes 5–7) and pGEM U (ura) (lane 8) plasmids were incubated with 30 µg of protein of GM 5757 extract for 1 (lanes 2, 5 and 8), 3 (lanes 3 and 6) and 6 h (lanes 1, 4 and 7). Quantification of data is shown in Figure 2B. At 1 h incubation time, the repair replication stimulated by 8-oxoG was undetectable while the single AP site was repaired on average 10-fold better than the single U (350 versus 35 c.p.m.). Repair of a single AP site reaches completion by this time (14,17). At 3 and 6 h incubation time, the ratio of repair incorporation between U and 8-oxoG substrates was 5.7- and 4.5-fold, respectively (mean: 5.1-fold). Hence, a single natural AP site was repaired on average 10×5 = 50-fold better than a single 8-oxoG. No background incorporation could be observed on pGEM T control plasmids incubated for 6 h under the same conditions used for pGEM U and pGEM U (ura) plasmids (lane 1).

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The capacity of GM 5757 human extracts to perform the PCNA-dependent BER pathway on 8-oxoG, U and natural AP site is shown in Figure 3. The experiment was performed using [32P]dCTP as labeled nucleotide and a restriction endonuclease treatment (HindII–PstI) that excises an 8mer fragment 3’ to the lesion. Due to the reduced efficiency of the pathway with respect to the one nucleotide insertion (14), a double amount of extract proteins (60 µg) was used. No repair incorporation could be observed at 3 or 6 h incubation time when using the pGEM 8-oxoG plasmid substrate (Figure 3A, lanes 1 and 2, and B, circles), thus suggesting that poor BER of 8-oxoG is exerted via the single nucleotide insertion pathway only. The PCNA-dependent repair of a single AP site (Figure 3A, lanes 5 and 6, and B, triangles) was 10.1- and 5.8-fold more efficient than that of a single U (Figure 3A, lanes 3 and 4, and B, squares) at 3 and 6 h incubation time, respectively.

Hazra et al. (18) have recently hypothesized the presence of an 8-oxoG glycosylase inhibitor or an 8-oxoG-binding protein in human cell extracts, after observing that low 8-oxoG repair could be enhanced by increasing markedly the molar concentration of the substrate. We therefore planned a repair replication experiment using as substrates the 22mer oligonucleotides employed for plasmid preparations (see Materials and methods), made duplex by annealing their complementary sequence (Figure 4A). Only short patch repair events can be detected with this kind of substrate (8) but its use allowed us to increase >800-fold the moles of substrate lesions per µg extract protein (from 4.9 fmol in the plasmid assay to 4.3 pmol in the oligonucleotide assay). To the high density of lesions corresponds a pronounced increase in the
levels of repair incorporation (note the different ordinate scale in Figures 2B and 4B). A total of 1200 ng of duplex 22mer oligonucleotide substrates carrying a single U (Figure 4A, lanes 1–4, and B, squares) or a single 8-oxoG (Figure 4A, lanes 5–8, and B, circles) was incubated with 20 μg protein of GM 5757 extract at 30°C for 0.5, 1, 3 and 6 h. Despite the substantial increase in the molar concentration of 8-oxoG, the repair efficiency of the latter lesion with respect to U remained essentially unchanged with repair replication of 8-oxoG undetectable during the first hour of incubation. At 3 and 6 h incubation time the repair signal stimulated by 8-oxoG was 2.3- and 4.9-fold lower than that stimulated by U, respectively, in reasonable agreement with what had been observed in the plasmid assay (Figure 2B). Hence, the relative efficiencies of repair of 8-oxoG and U by GM 5757 extracts do not seem significantly influenced by the molar concentration of the lesion.

The inefficient repair of 8-oxoG is linked to low 8-oxoG DNA glycosylase activity

The different repair replication efficiency of 8-oxoG and U by human cell extracts could be due to the different rates of the 8-oxoG glycosylase and U DNA glycosylase activities or to the different ability of the extracts to process the subsequent reaction intermediate [β-elimination-catalyzed cleaved strand (18,19) versus natural AP site (11)]. The glycosylase activities were determined by incubating GM 5757 extracts with linear duplex 22mer substrates carrying a single 8-oxoG or a single U for 0.25, 0.5, 1, 2, 3 and 6 h (Figure 5). The residual 8-oxodG and dU were then quantitated by HPLC coupled to
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Fig. 3. Repair of single 8-oxoG, U and natural AP site by human cell extracts: PCNA-dependent pathway. (A) pGEM 8-oxoG (lanes 1 and 2), pGEM U (lanes 3 and 4), pGEM U (ura) (lanes 5 and 6) and pGEM T (lane 7) plasmids were incubated with 60 µg protein of normal human extract GM 5757 at 30°C for 3 (lanes 1, 3 and 5) or 6 h (lanes 2, 4, 6 and 7). After the repair reaction, plasmids were purified and treated with *Hin*II–*Pst*I restriction endonucleases. (B) Quantification of repair incorporation (Net CPM of dNMP incorporated) on pGEM 8-oxoG (●), pGEMU (■) and pGEMU (ura) (▲). Average data from two independent experiments.

Fig. 4. The lesion concentration does not influence the efficiency of 8-oxoG repair. (A) A total of 1200 ng of 22mer duplex oligonucleotides carrying a single U (lanes 1–4; upper sequence) or a single 8-oxoG (lanes 5–8; lower sequence) was incubated with 20 µg of GM 5757 extract protein for the indicated times at 30°C in the presence of [32P]dTTP (lanes 1–4) or [32P]dGTP (lanes 5–8). Under these conditions, the number of lesions per µg protein extract is 872-fold higher than in the plasmid assay. After the repair reaction, the oligonucleotides were purified and resolved by polyacrylamide gel electrophoresis. For simplicity, only the sequences of damaged strands are shown. (B) Quantification of repair incorporation (net c.p.m. of dNMP incorporated) on oligo(8-oxoG) (●) or oligo(U) (■).
The poor repair of 8-oxoG was not linked to inability to process the β-elimination cleaved strand generated by the AP lyase activity of hOGG1. In contrast, a single β-elimination-generated cleaved AP site was repaired more efficiently than a natural, uncleaved AP site (Figure 6). In this experiment, the AP lyase incision was generated by treatment of pGEM U (ura) plasmids with *E. coli* nth protein (endonuclease III) rather than hOGG1. However, the two enzymes produce identical β-elimination incisions (18,19), being closely related and probably derived from a common ancestor (9). In conclusion, the poor repair of 8-oxoG in human cells was uniquely due to their low capacity to detach the oxidized base from the deoxyribosephosphate backbone. Whether this limited capacity may be linked to low expression of the hOGG1 gene or to the kinetic features of the hOGG1 protein will be the subject of further study.

Hazra *et al.* (18) have recently proposed that low hOGG1 activity could be due to the presence of an 8-oxoG-specific DNA binding protein after observing that, paradoxically, 8-oxoG-strand incision could be detected only when the concentration of the damaged substrate was increased 200-fold. However, we found no significant dependence of the repair efficiency on the molar concentration of the 8-oxoG substrate.

**Discussion**

Two studies have recently described the mechanism of repair of 8-oxoG by mammalian cell extracts (20,21). However, data on the efficiency of repair of this oxidized base, in comparison with other endogenous lesions are scant. BER is considered the prominent mechanism for 8-oxoG repair. A glycosylase specific for 8-oxoG repair (hOGG1) has been characterized (9,10) and other glycosylases with broad specificity, such as N-methylpurine-DNA glycosylase, also contribute to excision of 8-oxoG (22). In this paper, we show that BER of 8-oxoG by human cell extracts is poor, in comparison with two other endogenous, BER processed, lesions: U and the natural AP site. 8-oxoG was repaired approximately five times less efficiently than U and approximately 50 times less efficiently than the natural AP site. Less marked differences in the repair efficiency of the three lesions could be observed with Chinese hamster A8 cell extracts. Poor repair of 8-oxoG as compared with U and AP sites was also observed with transformed human cell lines (M.Bogliolo, O.Rossi and G.Frosina, unpublished data).

The natural AP site was the most easily repaired lesion, probably because the limiting glycolytic step was not required and incision could take place as the first step. The higher repair of U with respect to 8-oxoG reflected the different rates of U DNA glycosylase and 8-oxoG DNA glycosylase in human GM 5757 extracts. In particular, the time required by GM 5757 extracts to remove 50% of 8-oxoG from a synthetic duplex oligonucleotide was ~3-fold longer than that required for U (1 versus 0.36 h; Figure 5). Furthermore, 22% of 8-oxoG persisted unrepaired after 6 h incubation, when 100% U had been removed. These differences were smaller than those observed with the repair replication assay, where no repair synthesis could be detected on pGEM 8-oxoG during the first hour of incubation and a 4.5-fold difference was observed between pGEM 8-oxoG and pGEM U after 6 h (Figure 2), thus indicating that the efficiency of DNA repair synthesis is to a certain extent depending on that of base removal. It is indeed possible that DNA glycosylases and DNA polymerases functionally interact during BER, so as a partial deficiency in base removal also affects the efficiency with which polymerization is carried out with a final impairment effect.

Electrochemical detection or UV detection, respectively. The kinetics of removal of 8-oxodG (circles) was slower than that of dU (squares) with 50% of lesions removed after 1 and 0.36 h, respectively. After 6 h incubation, a significant portion (22%) of 8-oxodG lesions was still unrepaired while all dUs had been removed.

The two major human 8-oxoG and U DNA glycosylases (hOGG1 and hUNG) have different end products, the former being associated with an AP lyase activity leaving a β-elimination-catalyzed cleaved strand (18,19) and the latter being a monofunctional DNA N-glycosylase leaving a natural uncleaved AP site (11). The experiment in Figure 6 shows that GM 5757 extracts efficiently process an AP lyase-cleaved AP site. pGEM U (ura) was incubated with *E. coli* nth protein that cleaves AP sites leaving a β-elimination-catalysed incision identical to that produced by hOGG1 (18,19) [pGEM U (ura) (nth); Figure 6A inset, right lane] or nth buffer (Figure 6A inset, left lane). Either pGEM U (ura) (Figure 6A, lanes 1–3, and B, closed symbols) or pGEM U (ura) (nth) (Figure 6A, lanes 4–6, and B, open symbols) was then incubated with GM 5757 extracts (30 μg protein). No decrease in the repair replication efficiency could be observed on the AP lyase-nicked AP site, with respect to the natural AP site. In contrast, at early incubation times (0.25 h), the repair replication of the β-eliminated substrate (Figure 6A, lane 4, and B, open symbols) was 3.2-fold higher than that of the natural AP site (Figure 6A, lane 1, and B, closed symbols). At later incubation times (0.5 and 1 h), this ratio decreased to 1.5 and 1.0, respectively, the repair of pGEM U (ura) (Figure 6A, lanes 2 and 3, and B, closed symbols) reaching completion (14,17). No background incorporation was present on control pGEM T plasmids (lane 7). In conclusion, the different capacity of GM 5757 extracts to repair 8-oxoG and U was uniquely linked to the different rates of the glycolytic steps.

**Discussion**

Two studies have recently described the mechanism of repair of 8-oxoG by mammalian cell extracts (20,21). However, data enzymatically hydrolyzed and the content of 8-oxoG (µD) and dU (µD) determined by HPLC coupled to an electrochemical or ultraviolet detector, respectively. Data are the means ± SEM of three independent experiments.

Error bars are not indicated when smaller than symbols.
Fig. 6. Comparison of repair of a single natural AP site and a single AP site cleaved by the AP lyase activity of E. coli nth protein. (A) pGEM U (ura) plasmids carrying a single natural AP site (lanes 1–3; inset, left lane), pGEM U (ura) (nth) plasmids carrying a single natural AP site cleaved by the AP lyase activity of E. coli nth protein (lanes 4–6; inset, right lane) or control pGEM T plasmids (lane 7) were incubated with 30 µg protein of normal human extract GM 5757 at 30°C for 0.25 (lanes 1 and 4), 0.5 (lanes 2 and 5) and 1 h (lanes 3, 6 and 7). After the repair reaction, plasmids were purified and treated with XhoI–HincII restriction endonucleases. (B) Quantification of repair incorporation (net c.p.m. of dNMP incorporated) on pGEMU (ura) (m) and pGEM U (ura) (nth) (Δ). Average data from two independent experiments.

(Figure 4). The possible presence of an endogenous inhibiting factor for 8-oxoG repair probably requires further investigation.

Low repair of 8-oxoG could be due to the selective inactivation of hOGG1 or any cofactors specifically required for 8-oxoG repair, during the preparation of extracts by the procedure of Tanaka et al. (8,13). This remains an open question but we consider this possibility unlikely because hOGG1 shows no particular lability during purification procedures (10,18) and the low repair efficiency of 8-oxoG in comparison with AP sites, was also observed with extracts prepared by the procedure of Manley et al. (23) that are competent for complex DNA transactions such as nucleotide excision repair and transcription (data not shown, and ref. 21).

In two recent reports, Dianov et al. (20) and Fortini et al. (21), have shown that 8-oxoG is repaired mainly via the short patch pathway in HeLa cells. According to Fortini et al. (21), the AP site incision generated by a bifunctional glycosylase/AP lyase activity such as hOGG1 could be preferentially followed by single nucleotide replacement reactions, whilst the AP site incision generated by the sequential action of a monofunctional glycosylase, like 3-methyladenine-DNA glycosylase and of a 5′ AP endonuclease, like HAP1/APE, could be followed by long-patch, PCNA-dependent repair events that occur in competition with the predominant one gap filling reactions. The data presented here support this model, repair of 8-oxoG being entirely attributable to the single nucleotide insertion BER pathway, whereas repair of U, initiated by the monofunctional hUNG glycosylase, was in part accomplished via the PCNA-dependent pathway.

The poor repair of 8-oxoG found in mammalian cell extracts supports the hypothesis of a role of this endogenous lesion in mutagenesis and carcinogenesis (24).

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