Poly(ADP-ribose) polymerase-1 (PARP-1) is required in murine cell lines for base excision repair of oxidative DNA damage in absence of DNA polymerase β

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Running title: Repair of 8-oxoguanine in PARP-1⁻/⁻ Polβ⁻/⁻ 3T3 cells.
Abstract

Oxidative DNA base damage is mainly corrected by the base excision repair (BER) pathway which can be divided into two subpathways depending on the length of the resynthetized patch, either one nucleotide for short patch (SP-BER) or several nucleotides for long patch (LP-BER). The role of proteins in the course of BER processes has been investigated in vitro using purified enzymes and cell free extracts. In this study, we have investigated the repair of 8-oxo-7,8-dihydroguanine (8-oxoG) in vivo using wild-type, Polβ⁻/⁻, PARP-1⁻/⁻, and Polβ⁻/⁻PARP-1⁻/⁻ 3T3 cell lines. We used non replicating plasmids containing a 8-oxoG.C base pair to study the repair of the lesion located in a transcribed sequence (TS) or in a non transcribed sequence (NTS). The results show that 8-oxoG repair in TS is not significantly impaired in cells deficient in Polβ or PARP-1 or both. Whereas 8-oxoG repair in NTS is normal in Polβ-null cells, it is delayed in PARP-1-null cells and greatly impaired in cells deficient in both Polβ and PARP-1. The removal of 8-oxoG and presumably the cleavage at the resulting AP site are not affected in the PARP-1⁻/⁻Polβ⁻/⁻ cell lines. However, 8-oxoG repair is incomplete yielding plasmid molecules with a nick at the site of the lesion. Therefore, PARP-1⁻/⁻Polβ⁻/⁻ cell lines cannot perform 5’-dRP removal and/or DNA repair synthesis. Furthermore, the poly(ADP-ribosyl)ation activity of PARP-1 is essential for 8-oxoG repair in a Polβ⁻/⁻ context, since expression of the catalytically inactive PARP-1 (E988K) mutant does not restore 8-oxoG repair whereas an WT PARP-1 does.

Keywords: BER, 8-oxo-7,8-dihydroguanine, Polβ and PARP-1, 3T3 cell lines
Introduction

Reactive oxygen species, generated either endogenously by cellular metabolism or by exposure to environmental oxidants, induce DNA damages which have been implicated in human pathologies such as cancer, neurodegenerative diseases or ageing (1-4). An oxidatively damaged guanine 8-oxo-7,8-dihydroguanine (8-oxoG), is an important mutagenic DNA lesion due to its potential to mispair with adenine, thus generating G.C to T.A transversions. The biological significance of 8-oxoG is revealed by the spontaneous mutator phenotype of bacterial and yeast mutants impaired in 8-oxoG repair (5-9). In all organisms, 8-oxoG is primarily repaired by the base excision repair (BER) pathway which is the major process for the elimination of oxidative base damage, alkylation base damage and apurinic / apyrimidinic (AP) sites (10,11). In mammalian cells, BER of 8-oxoG is initiated by the action of the Ogg1 DNA N-glycosylase, which catalyzes the hydrolysis of the N-glycosyl bond linking damaged bases to the sugar-phosphate backbone generating AP sites. Ogg1 is also endowed with an AP lyase activity that can incise the phosphodiester bond immediately 3’ of an AP site yielding a 3’-terminal sugar phosphate (3’-dRP) (12-19). However, in the presence of the major AP endonuclease Ape1, AP sites resulting from the removal of 8-oxoG residues by Ogg1 are primarily processed by Ape1 which catalyzes the hydrolytic cleavage of the phosphodiester bond immediately 5’ to the AP site generating a 5’-terminal sugar phosphate (5’-dRP) (20,21). Afterwards, the 5’-dRP is removed by a dRPase activity associated with DNA polymerase β (Polβ) which simultaneously adds one nucleotide. The nick is finally sealed by DNA ligase III associated with the X-ray cross-complementing factor 1 (Xrcc1) (22-24). The entire process results in the removal of 8-oxoG and its replacement with a guanine and constitutes the Short-Patch Base Excision Repair (SP-BER) pathway. In repair proficient cells, SP-BER is thought to be the major repair pathway for 8-oxoG (25). Another form of BER, the Long-Patch BER (LP-BER) results in the removal of the DNA damage and replacement of 2-10 nucleotides extending 3’ to the lesion (26-31). In the course of LP-BER, the early steps of 8-oxoG repair are performed by Ogg1 and Ape1 as described for SP-BER. Then a DNA polymerase (Polβ, δ or ε) adds few nucleotides and
displaces the 5’-dRP residue generating a 5’-flap structure with a 5’-dRP end. The 5’-flap is removed by the Flap endonuclease 1 (Fen1) and a DNA ligase seals the nick. The role of different DNA polymerases in LP-BER in the wild type cellular context is unclear. Recently, DNA polymerase δ- or ε-dependent LP-BER have been reconstituted with purified human proteins (32). For efficient repair of a regular AP site, in addition to Ape 1 and DNA polymerase δ, the reaction assay required replication factor C (RF-C), proliferating cell nuclear antigen (PCNA), Fen1 and Ligase I (30). LP-BER is a minor pathway for the repair of 8-oxoG and regular AP sites in wild type cell free extracts (26,33). In contrast, LP-BER is thought to be the major pathway for the repair of reduced or oxidized AP sites (31).

Several studies pointed out Polβ as the major repair DNA polymerase in eukaryotes, involved in both SP- and LP-BER (24,30,34-36). Polβ lacks accessory functions such as 3’ to 5’ exonuclease, dNMP turn-over or pyrophosphorolysis (37-40). On the other hand, Polβ possesses a robust AP lyase activity that allows the removal of 5’-dRP in the course of BER (41). Polβ performs an essential function in development, since knock-out mice for Polβ are not viable (42,43). However, Polβ-null 3T3 cells are viable, but hypersensitive to methylating agents (39,44-46). The hypersensitivity to methylating agent can be suppressed by the expression of the AP lyase domain of Polβ (44). Although non essential, the poly(ADP-ribose) polymerase 1 (PARP-1) has been shown to interveine in the course of BER in living cells (47,48). PARP-1 is a nuclear protein found in proliferating tissues of eukaryotes with the exception of yeast (49,50). PARP-1 binds with high affinity DNA containing single-strand breaks. Upon binding to DNA strand breaks, PARP-1 catalyses the synthesis of poly(ADP-ribose) from NAD⁺ and covalently modifies several nuclear proteins involved in chromatin architecture (such as histones and lamin B) and in DNA metabolism (such as topoisomerases, DNA polymerases and BER factors). The automodification of PARP-1 induces its dissociation from DNA breaks and inhibition of its catalytic activity. PARP-1 and poly(ADP-ribosyl)ation are proposed to be critical for cellular processes such as DNA repair, transcription or energy depletion-induced cell death during inflammatory injury (see for review (50,51)). Evidence for the involvement of PARP-1 in BER was provided by the fact that PARP-1-null mice are hypersensitive to ionizing radiation and alkylating agents (52-54). Moreover, the physical interaction of PARP-1 with proteins such as
Polβ and Xrcc1 also points to its role in BER (55,56). Recently, PARP-1 was shown to bind with high affinity to BER intermediates harbouring a 5’-dRP (57). Furthermore, reconstitution of BER using purified proteins shows that PARP-1 stimulates two of the key steps of LP-BER: strand displacement synthesis by Polβ and 5’-flap cleavage by Fen1 (58). In addition, repair of AP sites is impaired in cell free extracts of PARP-1-null mice cell lines (46). This study also shows that PARP-1-null Polβ-null cell free extracts present a dramatic decrease in LP-BER when compared to PARP-1-null cells. Therefore, results with purified proteins and cell free extracts point to a role of PARP-1 in LP-BER.

To investigate the role of Polβ and PARP-1 in the course of BER in the cellular context, we measured the repair of 8-oxoG in murine cell lines (3T3s), either wild type (WT) or deficient in Polβ (Polβ−/−) or PARP-1 (PARP-1−/−) or in cells that present the two deficiencies, (PARP-1−/− Polβ−/−). We measured the repair kinetics for a single 8-oxoG.C base pair in these four cell lines using shuttle vectors that contain 8-oxoG in a transcribed sequence (TS) or a nontranscribed sequence (NTS) (59). Our results show that the repair of 8-oxoG in TS plasmid is not affected in wild type, Polβ-null, PARP-1-null and double PARP-1-null Polβ-null 3T3 cells. On the other hand, repair of 8-oxoG in NTS plasmid which is not affected in Polβ-null cells, occurs at a 2-fold reduced rate in PARP-1-null cells. Furthermore, 8-oxoG repair in the NTS plasmid is nearly completely abolished in cells deficient in both Polβ and PARP-1. The role of the poly(ADP-ribose) synthesis during BER is also discussed in this study.
Materials and Methods

Cell lines and culture conditions: Spontaneously immortalized 3T3 wild-type, homozygous PARP1−/−, homozygous Polβ−/− and double deficient PARP-1−/−Polβ−/− clones were established in DMEM, 4.5g/L glucose medium supplemented with 10% fetal bovine serum and 0.5% gentamycin (46). To obtain PARP1−/−Polβ−/− 3T3 cell lines corrected by expression of wild-type or mutant human PARP-1, double deficient cell lines were transfected with an empty vector pECV-23Xho or with a vector containing the cDNA encoding the wild type PARP-1 (pECV-PARP-1) (60) or the E988K mutant human PARP-1 protein (pECV-PARP-1E988K) (61). Transfectants were selected by growth in medium containing hygromycin at increasing concentration up to 400µg/ml. Single clones were isolated after 15 days, propagated in 12-well plates and analyzed for PARP-1 expression by western blotting.

Western blotting: Cell-free protein extracts were prepared from clonal isolates as previously described (59). The protein content was determined according to Bradford and 50 µg of protein were analyzed by 8 % SDS-PAGE and immunoblotting. For immunodetection, blots were incubated with anti-PARP-1 (1/4000, Montevideo) polyclonal antibodies. Blots were then probed with horseradish peroxydase-coupled secondary antibodies (goat anti rabbit 1/25000, Sigma) and immunoreactivity was enhanced by chemiluminescence according to the manufacturer (ECL, Pharmacia).

8-oxoG.C cleavage assay: Standard assay mixture (15 µl-final volume) contained 25 mM Tris-HCl pH 7.6, 2 mM Na2EDTA, 70 mM NaCl, 25 fmol of 5'-[32P]-labelled [8-oxoG.C] 34mer DNA duplex and 10 µg of cell-free protein extract (62).

In vivo 8-oxoG repair kinetics: Construction of pSΔoriSV-[8-oxoG.C] or pSΔ(ori-p)SV-[8-oxoG.C] used for repair kinetics has been described (59). pSΔoriSV-[8-oxoG.C] (TS) or pSΔ(ori-p)SV-[8-oxoG.C] (NTS) plasmids were transfected into semiconfluent 3T3 cell lines (Effectene reagent, Qiagen). Cells were incubated from 4 to 24 hours, harvested and plasmid DNA was recovered (63). Assays for removal kinetics of 8-oxoG were carried out using a
procedure previously described (59). Briefly, recovered extrachromosomal DNA was treated or not with 5ng of \textit{E.coli} Fpg protein (64) and analyzed on a 0.8% agarose gel containing ethidium bromide. Plasmid DNA was detected by southern blotting and quantification was done using a PhosphorImager (Molecular Dynamics). The repair of 8-oxoG at each time point corresponds to the ratio between covalently closed molecules (CC) after an Fpg treatment and the sum of (CC) molecules and open circles (OC). This assay requires that the integrity of the CC molecules is preserved during DNA extraction. Thus conversion into relaxed form (OC) of the plasmid DNA depends on Fpg treatment (59).

**Mutagenesis assay**: Transfection and plasmid extraction were performed as above. Recovered plasmid DNA was used to transform \textit{E.coli} strain (BH990) \textit{fpg mutY} by electroporation and transformants were selected by ampicillin resistance. The repair of 8-oxoG.C was monitored by NgoMIV digestion of plasmid DNA extracted from individual bacterial clones as previously described (65).

**Primer extension**: Recovered plasmid DNA was used as template for an “asymmetrical PCR amplification”. A 18mer, located at 80 bp from the site of the lesion and used as a primer was labelled in 5’–end by $\gamma$-[32P]-ATP and T4 polynucleotide kinase. Primer extensions were performed using an automatic thermocycler and \textit{E.coli} DNA polymerase I klenow fragment, 250 $\mu$M dNTPs in a reaction mixture containing 1mM DTT, 6.7 mM Tris-HCl pH 8.8, 6.6 mM MgCl$_2$ (Biolabs). Amplification conditions were : 29 cycles with 30 sec at 94°C, 1 min at 54 °C, 2 min at 72 °C. Reactions were quenched by denaturing loading buffer and the reactions products were resolved on a 6 % polyacrylamide/ 7M urea gel. A sequence of plasmid DNA using the unlabelled 18 mer primer was performed according to the standard protocol provided by the manufacturer (Sequenase 2.0 DNA sequence kit, USB) and migrated in parallel to the primer extension synthesis.

**Results**
Combination of Polβ and PARP-1 deficiencies abrogates 8-oxoG.C repair on NTS but not in TS.

To investigate the role of Polβ and PARP-1 proteins in the repair of 8-oxoG in the cellular context, we used 3T3 cell lines, wild type (WT), PARP-1<sup>+/−</sup>, Polβ<sup>+/−</sup> and PARP-1<sup>+/−</sup>Polβ<sup>+/−</sup> which have been previously characterized (46). The different 3T3 cells were transfected with non replicative plasmids that contain a single 8-oxoG.C base pair. Two constructs were used to allow repair analysis of 8-oxoG located in the same sequence context but with a different transcriptional status (59,66). One plasmid allows the analysis of 8-oxoG repair in a transcribed sequence (TS), whereas the other allows the analysis of 8-oxoG repair in the same sequence but nontranscribed (NTS) because of the deletion of the SV40 promoter (59,66). To monitor the repair of 8-oxoG, plasmid DNA was recovered after incubation in 3T3 cells and digested by the Fpg protein which specifically nicks DNA at the 8-oxoG.C base pair. In this assay, 8-oxoG repair is characterized by increasing amounts of covalently closed (CC) plasmid molecules that are resistant to cleavage by Fpg, indicating the replacement of the 8-oxoG.C pair with a G.C pair in DNA and sealing of the repair-induced nick or gap (59). Figure 1 (right panel) gives an illustration of different southern blots obtained with NTS constructs in each cell lines used. Figure 1 (left panel) shows a comparison of 8-oxoG.C repair kinetics in TS and NTS in WT and mutant 3T3 cell lines. Figure 1B shows that repair kinetics in Polβ-null cells are very similar to those obtained in WT cells (Fig.1A), independently of the transcriptional status of the lesion, TS or NTS. Therefore, DNA polymerase β is not required for the repair of 8-oxoG.C in the cellular context, indicating the action of other DNA polymerases. Figure 1C shows the repair kinetics of 8-oxoG.C in PARP-1-null cell lines. Again, there is no obvious difference between PARP-1<sup>+/−</sup> and WT with 8-oxoG.C in the TS status. In contrast, 8-oxoG.C repair on NTS is delayed in PARP-1-null cells, when compared to that observed in the WT cell line. However, full repair of 8-oxoG.C is observed at 24 hours in the PARP-1<sup>+/−</sup> cells (Fig1.C). These results suggest that the absence of PARP-1 impairs an efficient replacement of 8-oxoG with a guanine, even if the protein is not absolutely required, in the cellular context.

To further investigate of the role of Polβ and PARP-1, we analyzed the processing of 8-oxoG.C in a double deficient PARP-1<sup>+/−</sup>Polβ<sup>+/−</sup> cell lines. Figure 1D shows that 8-oxoG.C base
pair is efficiently repaired when located on a TS plasmid in both WT and PARP-1<sup>−/−</sup>Polβ<sup>−/−</sup> cell lines. In contrast, until eighteen hours after transfection, we do not observe detectable repair of 8-oxoG.C in NTS plasmid, whereas all molecules were repaired in WT cells. Finally, less than 10% of repair may be observed in the double knock-out cells 24 h after transfection (Fig. 1D). These results show that full repair of 8-oxoG.C is greatly impaired in NTS plasmid in cells lacking both Polβ and PARP-1 proteins.

**Removal of 8-oxoG in NTS is not affected in Polβ<sup>−/−</sup> PARP-1<sup>−/−</sup> double knock-out cells.**

The absence of repair of 8-oxoG.C in the NTS plasmid 12 hours after transfection in the PARP-1-null Polβ-null cells could be due to an impaired recognition and/or excision of 8-oxoG by the Ogg1 DNA N-glycosylase. Therefore, Ogg1 enzyme activity in crude extracts was assayed using as substrate a 34mer oligonucleotide containing a 8-oxoG.C base pair. Figure 2 shows that a cell free extract of the PARP-1-null Polβ-null 3T3 cells efficiently cleaves the [8-oxoG.C] duplex. Furthermore, western blotting analysis using anti-human Ogg1 antibodies reveals a normal expression of the murine Ogg1 in PARP-1-null Polβ-null cells (data not shown). These results strongly suggest that the removal of 8-oxoG is not globally affected. If 8-oxoG is efficiently processed by Ogg1, the plasmid DNA recovered from PARP-1-null Polβ-null cells should contain a nick at or near the site of the lesion. Figure 3 shows that NTS plasmid DNA recovered from PARP-1-null Polβ-null cells 12 hours after transfection, migrates as an open circle (OC) with and without Fpg-treatment. This result indicates that these plasmids have been nicked in the cell, presumably after removal of 8-oxoG. Unspecific cleavage of NTS plasmid in PARP-1-null Polβ-null cells is unlikely because TS plasmid transfected in the same cells is recovered as covalently closed circle (Fig. 3, bottom). The kinetics of strand cleavage of the NTS plasmid in PARP-1-null Polβ-null cells was compared to those of 8-oxoG repair in WT cells. Figure 4 shows that the kinetics of cleavage in PARP-1-null Polβ-null cells is very similar to that of full 8-oxoG repair in WT. To demonstrate the removal of 8-oxoG from the NTS plasmid in PARP-1-null Polβ-null cells, the recovered plasmid DNA was transformed into the fpg mutY mutant strain of E.coli. If 8-oxoG is still present in DNA, it has to induce specific G.C to T.A transversion during DNA replication in this bacterial strain (9,67). Therefore, after
amplification, the 8-oxoG-containing plasmids generate a population that contains one or two NgoMIV restriction sites. In contrast, plasmid without 8-oxoG generates a pure population containing a single NgoMIV restriction site as previously described (68). Our results show that only 1 out of 96 clones tested contained the 8-oxoG.C pair (data not shown). Taken together, these data strongly suggest that the removal of the 8-oxoG lesion in NTS is not affected by the deletion of Polβ and PARP-1 in the cell.

**Incomplete repair of 8-oxoG in double Polβ−/− PARP-1−/− cells in NTS results from a defect in 5’-dRPase and/or DNA repair synthesis activities.**

Repair assays with purified proteins or cell free extracts show that AP sites generated by Ogg1, after removal of 8-oxoG, are primarily incised at the 5’-side by Ape1 yielding 5’-dRP and 3’-OH (20,69). The results reported in this *in vivo* study show that removal of 8-oxoG in NTS or TS plasmid occurs normally in WT, PARP-1−/−, Polβ−/− and PARP-1−/−Polβ−/− double mutant. However, incomplete repair of 8-oxoG in NTS is observed in the double deficient cell line suggesting a defect in a late step in the course of the repair process. As an attempt to identify the repair intermediate that accumulates in PARP-1-null Polβ-null 3T3 cells, we performed primer extension studies using as template the NTS plasmid DNA recovered from the double deficient cell lines 12 hours after transfection. The principle of this assay is briefly described in Figure 5A. Extracted plasmid DNA is hybridized with a 5’-end [32P]-labeled 18mer primer specific to the strand containing the lesion. Afterwards, primer extension is performed using the Klenow fragment of *E.coli* DNA polymerase I. Figure 5B shows a strong arrest of polymerization with DNA recovered from PARP-1−/−Polβ−/− cells. In contrast, no polymerization arrest is observed using DNA recovered from WT or Polβ−/− (Fig. 5B). Location of the arrest band was determined by comparing primer extension and plasmid sequencing using the same primer. Sequence analysis indicates that the arrest band corresponds to an incorporation opposite to the original position of 8-oxoG in the plasmid DNA (Fig. 5B). This observation strongly suggest a cleavage of DNA by Ape1 at the site of the lesion resulting in the formation of a 5’dRP residue (Fig. 5A).
Poly(ADP-ribose) polymerase activity of PARP-1 is required for the 8-oxoG.C repair on NTS in vivo in Polβ−/− cells.

The results indicate that complete repair of 8-oxoG.C on NTS plasmid requires the PARP-1 in a Polβ-null background. To investigate the role of the poly(ADP-ribose) synthesis in the 8-oxoG.C repair process, we constructed 3T3 cell lines, pECV-PARP-1 and pECV-PARP-1E988K, that express either the wild-type human PARP-1 or a catalytically inactive PARP-1 mutant (E988K) in the PARP-1−/−Polβ−/− background. The E988K mutation inhibits PARP-1 activity without affecting its DNA binding capacity (61). Selected clones express the PARP-1 protein at a level similar to that of the wild type 3T3 cell line (Fig. 6). These cells lines were used to study the repair of 8-oxoG.C on NTS. As expected, the PARP-1−/−Polβ−/− cells expressing the WT PARP-1 (pECV-PARP-1) efficiently repair 8-oxoG.C on NTS (Fig. 7). In contrast, PARP-1−/−Polβ−/− cells expressing the mutant PARP-1 (pECV-PARP-1E988K), do not allow the repair of 8-oxoG.C on NTS (Fig. 7). Therefore, the poly(ADP-ribosyl)ation reaction performed by PARP-1 is required for the repair of 8-oxoG.C in vivo in absence of Polβ.
Discussion

The identification of proteins involved in the processes of base excision repair (BER) in mammalian cells is subject to intense investigation. Most studies are carried out using cell extracts or purified proteins (22-24,26,30,46,70,71,72). The role of “nonessential factors” such as PARP-1 is also subject of discussion, (for reviews, see (49,73,74)). In this work, we used an *in vivo* approach based on the transfection of monomodified plasmid in intact cells to study the repair of 8-oxoG in the cell context. In the last decade, shuttle plasmids have been used to analyze mutagenesis and repair in mammalian cell lines and mutagenic potency of specific lesions (75,76). In every cases, results gave a quite good preview of the process in genomic DNA. This system also allowed to study the repair of a 8-oxoG in either transcribed (TS) or nontranscribed (NTS) condition (59,77).

In this study, we investigated the repair of 8-oxoG in 3T3 cells deficient in PARP-1 or/and Polβ. Our data show that repair of 8-oxoG in transcribed sequence (TS) is not significantly affected by inactivation of Polβ and/or PARP-1 proteins. This last result points again for the occurrence of a specific DNA repair pathway associated with transcription (TCR) acting at oxidative DNA damage such as 8-oxoG in human and mice cells. In human cells, repair of 8-oxoG in TS requires TFIIH, XPG, CSB, BRCA1 and BRCA2 but is independent of XPA (66,78). In mouse cells, this pathway is dependent of Csb but independent of Ogg1, Polβ and PARP-1 ((59,77) and our study). In contrast, 8-oxoG repair in NTS requires BER proteins such as Ogg1 and the combination of Polβ and PARP-1 but not NER proteins (our study and (59,66,77)). PARP-1 has been reported to be a negative or positive regulator of transcription, by modifying and/or binding several transcription factors see for review (51). The absence of PARP-1 has no effect on TS, indicating that the function of PARP-1 in transcription is not related to DNA repair but is more likely to participate in the organization of the chromatin architecture (79).

Our study also show that Polβ, primarily involved in SP-BER, is not essential *in vivo* for 8-oxoG repair in NTS, in agreement with studies using cell free extracts (26,80). Presumably, in Polβ-deficient cells, other DNA polymerases such as Polδ and Polε would achieve DNA repair
resynthesis. The removal of the 5’-dRP normally carried out by Polβ 5’dRP lyase activity during SP-BER, could be achieved by Fen1 according to a LP-BER process (Figure 8). On the other hand, PARP-1 substantially influences repair of 8-oxoG located in NTS. Indeed, we observed delayed repair kinetics (about 2-fold) of 8-oxoG in NTS in PARP-1-/- cells compared to WT cells. This delayed repair, if it happens in the genomic DNA, might have dramatic consequences in the cells. Indeed, results obtained from “Comet” assays using PARP-1-/- cells treated with MMS also showed a delayed DNA strand breaks resealing causing cell growth retardation, G2/M accumulation and chromosome instability (48). Furthermore, inactivation of both Polβ and PARP-1 dramatically impairs the repair of 8-oxoG in NTS. The lack of 8-oxoG repair in PARP-1-null Polβ-null cells indicates that, at least, one step in the course of the BER processes cannot be performed in these cells. In the present work, we show that the removal of 8-oxoG by Ogg1, which is absolutely required to initiate BER of 8-oxoG in NTS, is efficiently performed in PARP-1-null Polβ-null cells. Furthermore, the rate of incision of 8-oxoG.C-containing NTS plasmid in PARP-1-/-Polβ-/- cells is very similar to the rate of full-repair in WT cells. These results strongly suggest that the removal of 8-oxoG by Ogg1 occurs normally in PARP-1-/-Polβ-/- cells. They also indicate that removal of 8-oxoG by Ogg1 is the rate limiting step in the course of BER in the cellular context as well as in cell free extracts. Since Ape1 is a very abundant and efficient AP endonuclease, the NTS plasmids recovered from PARP-1-/-Polβ-/- cells, most probably contain a nick at the site of the lesion. This is consistent with the primer extension analysis which shows a strong block at the site of the lesion. Therefore, we conclude that inactivation of both PARP-1 and Polβ does not impair early stages but rather late stage(s) in the course of BER of 8-oxoG, either the excision of the 5’-dRP or/and the DNA resynthesis.

The role of PARP-1 in the DNA polymerization step of LP-BER of AP site in cell free extract has been proposed (46). However, a direct involvement of this protein remains unclear, despite of physical interactions shown between PARP-1 and Xrcc1, Polβ and DNA ligase III (46,55,56,81). Recently, Lavrik et al. (57) have demonstrated the high affinity of PARP-1 for a BER intermediate harboring a nick with a 5’-dRP end. Theses studies were recently extended to show that PARP-1 stimulates FEN1 and Polβ during strand displacement synthesis in a reconstituted system (58). Therefore a defect in PARP-1 would lead to a reduction of LP-BER.
efficiency. Our in vivo study shows that 8-oxoG repair in NTS is delayed in PARP-1-null cells and abolished in PARP-1 null Polβ null cells. In PARP-1-null cells, removal of the 5’-dRP and DNA repair synthesis would be SP-BER and dependent upon Polβ (Figure 8). However, the delay observed in 8-oxoG repair led us to conclude that Polβ is rate limiting in PARP-1-deficient cells. In PARP-1−/− Polβ−/− cells we may think that the 5’-dRP is processed at a very slow rate because of Polβ inactivation and a low level of activity or expression of Fen1 to act in absence of PARP-1 (31,74). Alternatively, the recruitment of replicative DNA polymerase may be very inefficient in the absence of PARP-1. Our results also indicate that poly(ADP-ribosyl)ation of PARP-1 or other factors is essential in that specific context. Other studies suggested a correlation between PARP-1 automodification and improved DNA repair (82,83). Poly(ADP-ribosyl)ation might be important either for the recruitment of BER proteins or for its dissociation from the nick DNA. In addition, poly(ADP-ribosyl)ation activity missing was shown to be the cause of the hypersensitivity of PARP-1−/− cell lines to ionising radiation and alkylating agents (71,84).

In conclusion, the efficient repair of 8-oxoG observed in WT cells may reflect the requirement for PARP-1 and Polβ in BER of 8-oxoG in distinct but overlapping subpathways each of which can compensate for loss of the other (Figure 8). Our work is the first demonstration for a role of PARP-1 and poly(ADP-ribosylation) in late stage(s) in the course of BER of 8-oxoG in the cellular context.
Figure legends

Figure 1: Kinetics of 8-oxoG.C repair in 3T3 cell lines. The non replicating shuttle vectors pSΔoriSV-[8-oxoG.C] (TS) and pSΔ(ori+p)SV-[8-oxoG.C] (NTS) were transfected into: (A) WT, (B) Polβ−/−, (C) PARP-1−/− or (D) PARP-1−/−Polβ−/− 3T3 cell lines. Plasmid DNA was recovered after 4- to 24-h incubations. Extracted plasmid DNA was treated by Fpg, migrated on a agarose gel containing ethidium bromide. DNA migration was observed after Southern blotting and the nicked (OC) and covalently closed (CC) plasmids were quantified using a PhosphoImager (Right panel) show representative southern blottings of pSΔ(ori+p)SV-[8-oxoG.C] in Polβ−/−, PARP-1−/− and PARP-1−/−Polβ−/− 3T3 cell lines. (Left panel) show the percentage of repair corresponding to the ratio of covalently closed molecules to the total amount of recovered plasmid DNA. Experimental values are the average of 2 or 3 blots resulting from independent transfections with two independent preparations of monomodified plasmid DNA. Error bars are shown.

Figure 2: Cleavage of a 34 mer [8-oxoG.C] duplex by crude cell-free protein extracts of 3T3 cell lines. A 34 mer oligodeoxyribonucleotide containing a single 8-oxoG was 5′[32P]-labelled and hybridized with a complementary strand yielding a duplex containing a cytosine opposite 8-oxoG. The [8-oxoG.C] duplex was incubated with 10 micrograms of crude cell-free extracts from WT, PARP-1+/−, Polβ+/-, or double PARP-1+/−Polβ+/- 3T3 cell lines. The assay was performed at 37°C for 15 min and the reaction products were analyzed in a 20% denaturing PAGE. Control (-) is [8-oxoG.C] duplex incubated with buffer. The substrate corresponds to the 34-mer and the product to the 16-mer obtained after successive cleavages by Ogg1 DNA glycosylase and AP endonuclease-containing extract.

Figure 3: Visualization of the pSΔoriSV-[8-oxoG.C] (TS) and pSΔ(ori+p)SV-[8-oxoG.C] (NTS) plasmid molecules after 12 h incubation in WT and PARP-1+/−Polβ+/- cell line. Extracted DNA was treated or not by Fpg, migrated on an agarose gel containing ethidium bromide and revealed after Southern blotting. As controls, migration of open (OC) and covalently closed molecules (CC) are indicated.
Figure 4: Kinetics of cleavage of pS(ori+p)SV-[8-oxoG.C] (NTS) plasmid in PARP-1⁻/⁻ Polβ⁻/⁻ cell line and full repair of the same plasmid in WT cell line. pS(ori+p)SV-[8-oxoG.C] construct was transfected in PARP-1⁻/⁻ Polβ⁻/⁻, recovered after 2- to 12-h incubation and analyzed by Southern blotting without previous Fpg treatment. In parallel, repair kinetics of the constructs were obtained as described in Figure1. Curves obtained for the double deficient (○○○) and WT (■■■) lines correspond to the quantitative analysis of 2 to 6 Southern blots by time point. Error bars are shown.

Figure 5: Primer extension study using NTS plasmid recovered from deficient and WT cell lines. pS(ori+p)SV-[8-oxoG.C] (NTS) construct was transfected in 3T3 cell lines deficient in PARP-1⁻/⁻ Polβ⁻/⁻ and Polβ⁻/⁻ or WT and recovered after 12 h incubation. (A) Rational of the study: X indicates the site of the lesion, it may be an intact guanine if the repair is complete or a 8-oxoG/nick/gap if it is not (arrows show the incision site by Ogg1 and Ape1). (B) Analysis of primer extension products on 6 % denaturing polyacrilamide gel. A sequence obtained using the same primer shows the complementary strand of the sequence containing the lesion and the location of the arrest of polymerization. NgoMIV restriction site is indicated. C is the base opposite the lesion.

Figure 6: Identification of stably PARP-1-expressing clones. PARP-1⁻/⁻ Polβ⁻/⁻ cell lines were transfected with a mock vector, a vector containing the cDNA encoding a WT PARP-1 cDNA or a vector containing the cDNA encoding a mutated PARP-1 gene (E988K). Total cell extracts were prepared and analyzed by western blotting using anti-PARP-1 polyclonal antibodies.

Figure 7: Kinetics of 8-oxoG.C repair in PARP-1⁻/⁻ Polβ⁻/⁻ 3T3 cell lines expressing WT and mutated PARP-1. The non replicating shuttle vector pS(ori+p)SV-[8-oxoG.C] (NTS) was transfected into PARP-1⁻/⁻ Polβ⁻/⁻ 3T3 cell lines, recovered after 6- and 12-h incubations and analyzed for the repair of 8-oxoG.C base pair. (Left) Southern blotting after transfection in the different clones and treatment by Fpg as described in Figure 1. Control lanes
« C » corresponds to monomodified plasmids incubated with (+) or without (-) Fpg. (Right)
Quantitative analysis of the Southern blots. Experimental values are the average of two blots resulting from two transfections from independent preparations of monomodified plasmid DNA.
Errors bars are shown.

**Figure 8 : A scheme for an efficient 8-oxoG repair in WT cell lines : role of Polβ and PARP-1 proteins.** After removal of the lesion by Ogg1 DNA glycosylase and cleavage of the resulting AP site by Ape1 endonuclease, two subpathways would co-exist *in vivo*, one SPR-Polβ dependent (8.1) and the other LPR-PARP-1 dependent (8.2). An overlapping role of PARP-1 and polβ would be when PARP-1 would accelerate the recruitment of the DNA polymerase β. The 5’dRP only or a short oligonucleotide containing the 5’dRP would be removed, depending on the subpathway used, and BER would be achieved. G0 corresponds to the lesion.
Acknowledgements

We thank, Dr Josiane Ménissier-de Murcia for the establishment of the 3T3 cell lines and Drs Lionel Gellon, Stephanie Marsin and J.Pablo Radicella for helpful discussion. This work was supported by CNRS and CEA and by the Association pour la Recherche sur le Cancer (ARC) to S.B (N°5432). This work was also supported by funds from CNRS, Association pour la Recherche Contre le Cancer, Electricité de France, Ligue Nationale Contre le Cancer and Commissariat à l’Energie Atomique to GdM.
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Figure 1

PARP-1<sup>-/-</sup>

PARP-1<sup>-/-</sup> Polβ<sup>-/-</sup>
Figure 2

WT
PARP-1−/−
Polβ−/−
PARP-1−/− Polβ−/−
Figure 3

### Table 1: DNA Repair Activity

|       | Control DNA | PARP-1<sup>-/-</sup> Polβ<sup>-/-</sup> | WT |
|-------|-------------|----------------------------------------|-----|
| NTS   | +Fpg -Fpg   | +Fpg -Fpg                              | +Fpg -Fpg |
|       | OC →        | OC →                                   | OC → |
|       | CC →        | CC →                                   | CC → |
| TS    | +Fpg -Fpg   | +Fpg -Fpg                              | +Fpg -Fpg |
|       | OC →        | OC →                                   | OC → |
|       | CC →        | CC →                                   | CC → |
Figure 4
Figure 6

WT
Polβ−/
PARP-1−/
PARP-1−/ Polβ−/
pECV-
PARP-1_{E988K}
pECV-PARP-1
pECV-XhO

Figure 6
DNA glycosylase (Ogg1)

AP endonuclease (Ape1)

AND/OR

PARP-1

DNA polymerase β

DNA polymerase β

DNA Polymerase δ, ε

Fen1

Complete repair (8.1 SP-BER)

Complete repair (8.2 LP-BER)

Figure 8
Poly(ADP-ribose) polymerase-1 (PARP-1) is required in murine cell lines for base excision repair of oxidative DNA damage in absence of DNA polymerase β
Florence Le Page, Valérie Schreiber, Claudine Dherin, Gilbert de Murcia and Serge Boiteux

*J. Biol. Chem.* published online March 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212905200

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