DNA polymerase β imbalance increases apoptosis and mutagenesis induced by oxidative stress

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Abstract Oxidative stress has been proposed to be one of the major causes leading to the accumulation of mutation that is associated with the initiation and progression of cancers. Elevated expression of DNA polymerase β, an event found in many human tumors, has been shown to generate a mutator phenotype. Here, we demonstrated that overexpression of DNA polymerase β strengthens the mutagenicity of oxidative damages, concomitantly with a higher cellular sensitivity and increased apoptosis. Deregulated expression of DNA polymerase β could represent a predisposition factor for mutagenic effects of oxidative stress and thus have implication in the generation and/or evolution of cancer. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: DNA polymerase β; Oxidative stress; Mutagenesis; Apoptosis; Genetic instability; Cancer

1. Introduction

DNA polymerases are key enzymes involved in most of cellular mechanisms maintaining the genomic integrity (i.e. replication, repair, recombination). In somatic eukaryotic cells, DNA polymerase β (Pol β) is the base excision repair (BER) polymerase which is expressed at a constant low level throughout the cell cycle [1] and is inducible by some genotoxic agents [2]. Because of its error-prone features, i.e. a low fidelity in replicating DNA in vitro [3], and a poor ability to discriminate nucleotides at the level of binding [4], deregulation of the expression of Pol β may be important in the predisposition of cell to genetic instability. The mutator phenotype concept has been extensively associated to cancer. It is well accepted now that cancers can result from the accumulation of mutations at the nucleotidic or chromosomic level which increases the probability to alterate genes that directly control and maintain the genetic information. High levels of Pol β has been found in prostate, breast and colon cancer tissues [5] as well as in ovarian tumors [6]. Previously, we demonstrated that the increase in Pol β expression is sufficient to lead to the accumulation of many mutations and we proposed that excess Pol β can compete with error-free DNA polymerases in DNA transactions such as DNA repair, synthesis or recombination [7]. By taking into account overall observations, we thus suggest a possible involvement of Pol β in some tumorigenesis processes.

Among the multiple mechanisms involved, we previously hypothesized that incorporation of oxidized nucleotides, which are produced endogenously, during the oxidative metabolism of the cell or as a consequence of exposure to radiations or to exogenous genotoxic chemicals, may play a role in this mutagenic process [7]. Oxidative stress generates different products including the hydroxyl ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) radicals that exert their effect by attacking DNA directly as well as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) which result in the formation of $^{\bullet}OH$ by the Fenton reaction using metal ions as activators [8]. Although all cellular macromolecules are subject to damage by reactive oxygen species (ROS), the primary deleterious consequences of oxidative stress probably arise from damage to nucleotides into the DNA or into the pool of free nucleotides. Because Pol β has the ability to incorporate nucleotide analogs [9] and also exhibits in vitro the lowest discrimination against mutagenic analogs of dGTP modified by oxidative processes [4], we tested here the possibility that oxidative stress could favor the mutator phenotype induced by high levels of Pol β. Using a Chinese hamster ovary (CHO) cell model consisting of an isogenic couple of recombinant cells differing only from a constant overexpression of Pol β, we studied the cellular response to H_2O_2 treatment, which produces ROS, that have the potential to damage DNA. Excess Pol β resulted in cellular hypersensitivity to H_2O_2 and was correlated to an increase in the apoptotic response. Besides, surviving cells overexpressing Pol β accumulated mutations in a very important extent, driving to the hypothesis that oxidative stress could be one of the ways by which Pol β exerted its mutagenic effect.

2. Materials and methods

2.1. Cell culture and survival analysis

Pol β-overexpressing plasmid pUTpolβ was constructed following a procedure previously described [9] to be stably inserted into the genomic DNA of transfected CHO cells. Cells were grown in monolayer in modified Eagle's medium supplemented with glutamine, 10% fetal calf serum, and pen/strep. All incubations were at 37°C in a humidified 5% CO_2 atmosphere. Sensitivity of the cell lines was determined

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Abbreviations: Pol β, DNA polymerase β; BER, base excision repair; CHO cell, Chinese hamster ovary cell; Sh cells, CHO cells transfected with the control DNA expression vector carrying the *ble* gene alone, conferring resistance to zeocin; Pol β::Sh cells, CHO cells transfected with the DNA expression vector overexpressing Pol β and conferring resistance to zeocin

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by clonogenic assay. Cells (500 per dish) were plated in 60 mm diameter dishes and let attach overnight. Then cells were treated 30 min in PBS with increasing concentrations of H_2O_2. After 6 days post-treatment incubation, colonies were fixed with methanol, stained with cresyl violet and colonies of more than 50 cells were scored and the percentage of survival was determined. Each experiment was performed in duplicate.

2.2. Detection of apoptosis

For cell death analysis, cells were treated 30 min with 100 μM H_2O_2 in PBS, as described above. Floating and attached cells were harvested at 24, 48 and 72 h after treatment.

Cells (3×10^6) were pelleted, rinsed with PBS and digested overnight at 37°C in 1 ml of lysis buffer (10 mM Tris–HCl, pH 8.0, 100 mM EDTA, 0.5% SDS) and 0.5 mg/ml proteinase K. The lysate was extracted twice with phenol/chloroform (25:24). DNA was precipitated with 0.04 volume of 5 M NaCl and 0.6 volume of isopropanol, recovered by centrifugation, dissolved and treated with RNase A for 1 h at 37°C. 1 μg of DNA was electrophoresed in 1.8% agarose gel. Gel was stained with ethidium bromide (1 μg/ml, 30 min), destained in distilled water and DNA pattern was photographed under ultraviolet illumination.

2.3. Quantification of apoptosis

The morphology of apoptotic cells was examined 24, 48 and 72 h after H_2O_2 treatment (100 μM) by staining cells with the fluorescent dye, DAPI (Sigma). Cells (2×10^5) were pelleted, rinsed with PBS, fixed with formaldehyde 3.7% and spread out on a microscope slide by centrifugation. Then slides were rinsed with PBS and incubated with DAPI (1 μg/ml). Fluorescence observation was performed with a Zeiss Axioskop microscope; at least 200 cells were scored for each experimental point.

2.4. Mutagenesis analysis

For determination of H_2O_2-induced mutagenesis, cells were treated with 50 μM H_2O_2, as described above, and let grow for 1 week. Then replica cultures were plated at the density of 10^6 cells and exposed to 20 μM 6TG-containing media in order to determine the number of hypoxanthine phosphoribosyl transferase (HPRT) mutants. After 1 additional week, macroscopic colonies were scored and mutation frequencies were calculated by correcting for plating efficiency and H_2O_2 survival.

3. Results

3.1. Overexpression of pol β promotes hypersensitivity to H_2O_2

The two cell lines used in this study are two isogenic CHO cell lines, which differ only by the overexpression of Pol β in about a three-fold extent (data not shown). The *Pol β::sh* cell line has been constructed by stably transfecting a plasmid containing *pol β* cDNA fused in frame with the bacterial

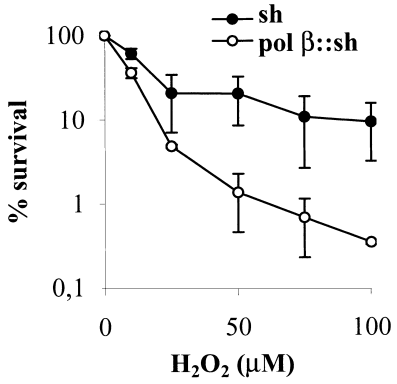

Fig. 1. Sensitivity of *sh* (closed circles) and *pol β::sh* (open circles) CHO cell lines to hydrogen peroxide. Survival is expressed as the relative plating efficiency of treated cells versus control cells. Results are the mean ± S.D. of three separate experiments.

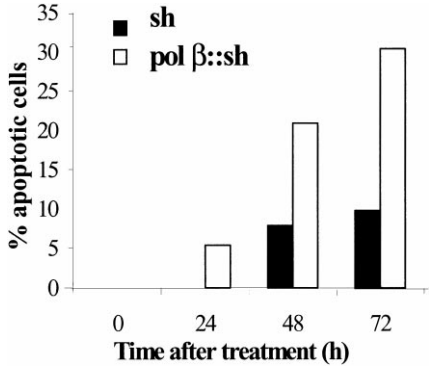

Fig. 2. Apoptotic cell response of *sh* (solid bar) and *pol β::sh* (open bar) CHO cell lines after hydrogen peroxide treatment. Cells were treated 30 min at 37°C with 100 μM of H_2O_2 in PBS and harvested 0, 24, 48 and 72h after treatment in order to perform nuclear morphology analysis (DAPI). At least 200 nuclei were examined and the percentage of apoptotic cells calculated as the ratio of nuclei bearing apoptotic bodies versus total stained nuclei. The data are from two independent experiments with a single result of a typical experiment shown.

Sh::ble gene that confers resistance to the broad-spectral zeocin xenobiotic of the phleomycin family. The fusion did not alter Pol β expression [9].

In order to investigate the cellular response to hydrogen peroxide (H_2O_2), we performed a cytotoxic analysis of these cell lines by clonogenic assay, allowing to measure the ability of treated cells to form colonies. Cells were treated with increasing concentrations of H_2O_2 and a clear hypersensitivity of cells overexpressing Pol β relative to control cells was observed (Fig. 1). The sensitivity to H_2O_2 is increased by five-fold in *pol β::sh* cells (DL10 = 20 μM H_2O_2) as compared to the *sh* cells (DL10 = 100 μM H_2O_2), demonstrating that Pol β interferes with the cellular response associated with oxidative stress.

3.2. Overexpression of pol β enhances H_2O_2-induced apoptosis

We firstly characterized H_2O_2-induced cell death as apoptosis in both *sh* and *pol β::sh* CHO cells lines in response to H_2O_2 by analyzing DNA fragmentation; 24, 48 and 72 h after H_2O_2 treatment, lysates from cells treated with 100 μM H_2O_2 for 30 min were resolved by agarose gel electrophoresis and stained with ethidium bromide. DNA cleavage can be observed earlier (48 h post-treatment) in the case of *pol β::sh* cells as compared to the control cell line (72 h post-treatment) (not shown). Apoptosis was further confirmed by analysis of chromatin condensation revealed by fluorescence microscopy (Fig. 2). 24 h after H_2O_2 treatment, DAPI-stained *pol β::sh* cells already displayed characteristics features of apoptosis such as condensed chromatin and subnuclear bodies (Fig. 2B). *sh* cells showed a slight apoptotic response (8%) 48 h after H_2O_2 treatment as compared to *pol β::sh* cells which have a higher apoptosis index (21%). 72 h after treatment, we observed 10% and 31% of apoptotic cells in *sh* and *pol β::sh* cells, respectively. Taken together, these results demonstrate that deregulation of Pol β expression increases by three-fold the apoptotic response induced by oxidative stress.

3.3. Pol β potentiates mutagenesis induced by H_2O_2

To further analyze the genotoxic effects of H_2O_2 in presence of excess Pol β, we measured the mutation frequency on a

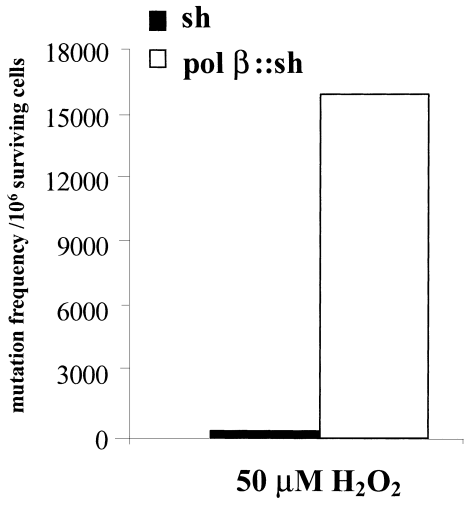

Fig. 3. Hydrogen peroxide-induced mutagenesis in *sh* (solid bar) and *pol β::sh* (open bar) CHO cell lines. For determination of hydrogen peroxide-induced mutagenesis, cells were treated at 50 μM for 30 min in PBS, allowed to grow for 1 week before plating at 10^6 cells in 6-TG-supplemented medium and grown for 1 additional week. Next, plates were fixed, stained with cresyl violet, and 6-TG^R mutant colonies larger than 50 cells were counted.

target gene, HPRT, involved in the purine-salvage pathway. *sh* and *pol β::sh* cells were exposed to 50 μM H_2O_2 and after 1 week cells were plated in presence of 6-thioguanine (6-TG) in order to select mutant clones, revealed as 6-TG-resistant colonies. The mutation frequencies measured for *sh* and *pol β::sh* cells were 3.3×10^{-4} and 160×10^{-4}, respectively (Fig. 3). This significant 50-fold increase for *pol β::sh* cells in production of mutations compared to *sh* cells demonstrates that genetic instability provoked by oxidative stress could be strengthened by a high expression of Pol β into the cell. This data also suggests that the mutator phenotype induced in these cells by excess Pol β could be the result of hyperactivity of endogenous oxidative damage.

4. Discussion

In the course of the identification of factors responsible for the initial mutagenic events that create a mutator phenotype leading to carcinogenesis, the role of oxygen species-mediated DNA damage has been extensively investigated (for review [10–13]. The ROS are generated in vivo and are products of metabolic processes such as mitochondrial respiration and cytochrome P-450 reactions [14,15]. They are also produced in response to xenobiotic agents and ionizing radiations. Oxidative stress results in alteration of a variety of cellular components and particularly DNA. Free radicals produce a number of lesions in DNA such as oxidized bases, sugar lesions, single-strand breaks, double-strand breaks, abasic sites and DNA protein crosslinks by a variety of mechanisms [16]. In response to these genetic modifications, the cell has developed different mechanisms of repair including the BER which reconstitutes genomic integrity. 8-oxoG, one of the most common oxidative adducts in mammalian DNA [17], can be formed directly into DNA by attack of $^{\bullet}OH$, but oxidation of guanine also occurs in the cellular nucleotide pool. Irrespective of how it is generated, 8-oxoG is a potent mutagenic substrate for DNA synthesis, since it can pair with adenine as well as cytosine in DNA, at almost equal frequencies [18]. In this case, both types of transversions, A→C and G→T, would be induced [19].

Previously, we demonstrated that the cellular overexpression of Pol β resulted in the apparition of a mutator phenotype [20], and we proposed that this could result from enhancement of genotoxic effects of oxidized lesions produced endogenously [7] by favoring their incorporation as modified nucleotides into DNA. Indeed, we have also shown that excess Pol β in HeLa cell extracts facilitates the incorporation of radiolabeled 8-oxo-dGTP into plasmidic DNA harboring a SV40 origin (unpublished data). Furthermore, Pol β has been shown to perform mutagenic translesion synthesis throughout 8oxoG and to produce misincorporations in the neighboring of the lesion [21,22]. To test this hypothesis, we treated the cells with H_2O_2 as generator of oxidative damages. We observed a very important increase in accumulation of mutations for cells overexpressing Pol β compared to control cells. This result shows that excess Pol β plays a key role in the generation of mutations in response to oxidative stress.

To date, there are no direct evidence for a link between mutagenesis by ROS and initiation of any human cancer or accumulation of mutations during the progression of cancer, but a lot of arguments seem to correlate these events (for review [11,12,23]). Numerous studies have been conducted on the levels of oxidized bases in tumor tissue or in chronic inflammatory states. One example is the involvement of oxidative damage in the development of breast cancer. Malins and Haimonat reported an association between oxidized DNA bases and breast cancer, demonstrating a nine-fold elevation in the levels of 8oxoG, 8oxoA and a formamido pyrimidine, as free nucleotides, in tumor tissue compared with surrounding normal tissue [24,25]. Besides, Pol β level of expression has been found higher in several cancers tissues than in normal adjacent tissue, for example in breast adenocarcinoma [5]. Presence of tumoral cells with abnormal elevated quantity of Pol β could thus accelerate the evolution of cancer.

Also, bursts in the generation of ROS are frequently associated with inflammatory processes and aging [23]. The association of such phenomenons with high levels of Pol β should increase the risk of apparition of cancer.

We also observed a higher apoptotic response when Pol β is present in excess in the cell. This increased apoptosis can be seen as a mechanism of elimination of cells harboring too many DNA damage and/or mutations. In this case, apoptosis can be considered as a guardian of cellular homeostasis by the elimination of cells which could harbor potential mutations deregulating cell growth and ultimately conducting to cancer. In our cell system, the data demonstrated that apoptosis can not eliminate all the cells harboring mutations. Abnormal cellular response to DNA damage can lead to the selection of cells with a greater propensity for neoplastic transformation.

Our work identify Pol β as a strong predisposition factor for mutagenic and carcinogenic effects of oxidative process. High levels of this enzyme in cancer cells may considerably accelerate the genotoxicity of oxidative stress.

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