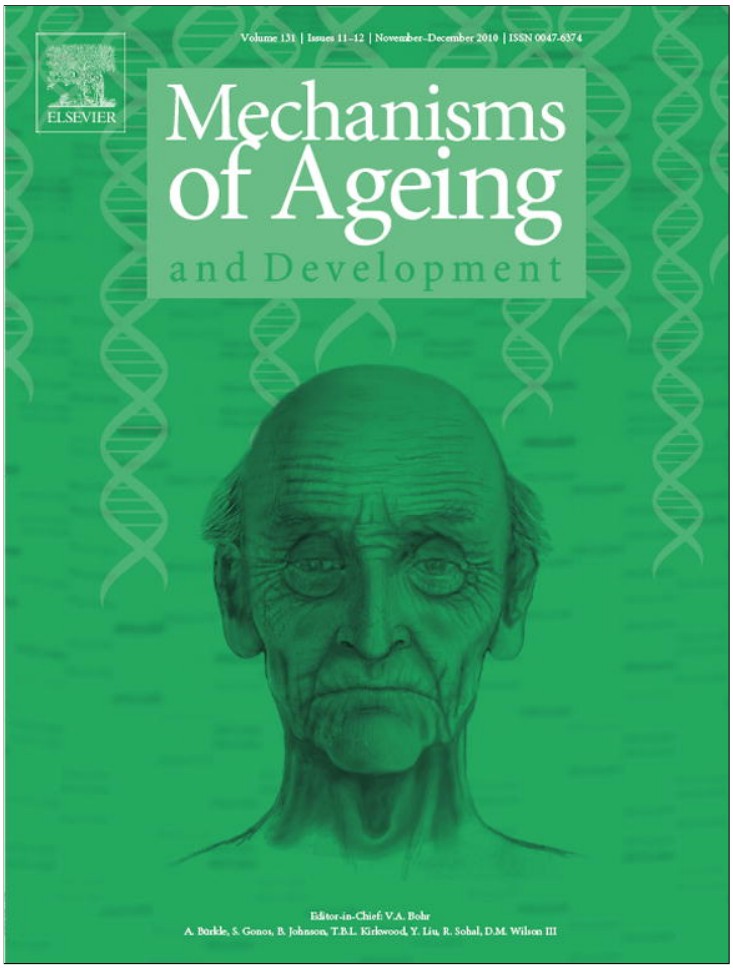

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Age-associated modifications of Base Excision Repair activities in human skin fibroblast extracts

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ABSTRACT

Base Excision Repair (BER) is the predominant repair pathway responsible for removal of so-called small DNA lesions such as abasic sites (AP site), uracil (U), 8-oxo-7,8-dihydroguanine (8oxoG), thymine glycol (Tg). In this study, we investigated effect of aging on excision efficacy of several endogenous base lesions and AP sites using an *in vitro* multiplexed fluorescent approach on support (parallelized oligonucleotide cleavage assay). Human fibroblasts nuclear extracts from 29 donors of different ages were characterized in their ability to simultaneously excise the different lesions. Clearly, three different groups of lesions emerged according to the efficiency of their cleavage: one exhibited very high cleavage efficiency (AP sites and U paired with G), one showed intermediate cleavage efficiency (U paired with A and Tg). The third group included 8oxoG, A paired with 8oxoG, T at CpG site and hypoxanthine (Hx) and displayed poor repair.

Aging was significantly associated with modification of excision efficiency for AP sites, uracil, Tg and 8oxoG. Repair rate decreased for the first three lesions and the most drastic effects were observed for repair of U:A.

Surprisingly, excision of 8oxoG increased with aging suggesting a completely different regulation or adaptation for the initiation step of this related specific repair pathway.

1. Introduction

Base Excision Repair (BER), which specificity relies on recognition/excision of base damage by glycosylases and AP endonuclease, is the predominant repair pathway responsible for removal of spontaneous damage, reactive oxygen species (ROS) induced DNA lesions and exogenously or endogenously generated alkylated bases. Among the most common formed base lesions, but not restricted to, are 8oxoG primarily recognized by oxoguanine DNA glycosylase (OGG1), uracil removed by uracil DNA glycosylase (UNG), thymine glycol (Tg) cleaved either by human Endonuclease III homolog (NTH1) or by NEIL1. The mechanism of BER involves the initial action of DNA glycosylases followed by the processing of the resulting abasic site either by the innate AP-lyase activity of the bifunctional glycosylase or by the apurinic/apyrimidic endonuclease APE1 that incises the DNA strand at the 5′-phosphate. Then polymerase and ligase mediate the final DNA synthesis/ligation steps. Recognition by glycosylases of BER in humans is rather specific although a redundancy in substrate specificity exists (Lindahl and Wood, 1999; Nilsen and Krokan, 2001).

Accumulation of potentially mutagenic DNA lesions is at the origin of alteration of the genome and the resulting genetic instability leads to cell aging and cancer. Because of its role to minimize mutagenesis, BER efficacy, in relation with aging, is a subject of investigation. Several lines of evidence indicate that BER-related functions decline with age. However most available studies are conducted in mouse cells and tissues and very few extensive studies exist in humans (Cabelof, 2007; Xu et al., 2008). In addition, no distinction between initiation steps of BER and overall BER capacity is usually made (Cabelof, 2007). Recently, evidences for an adaptation of repair response to increased oxidative damage were presented. First evidence concerned the increase of 8oxoG-incision activity with age in mouse liver mitochondria (de Souza-Pinto et al., 2001) suggesting an up-regulation by oxidative stress. Second

Abbreviations: BER, Base Excision Repair; NER, Nucleotide Excision Repair; AP site, abasic site; U, uracil; 8oxoG, 8-oxo-7,8-dihydroguanine; Tg, thymine glycol; Hx, hypoxanthine; ROS, reactive oxygen species; AAG, Human alkyladenine DNA glycosylase; APE1, apurinic/apyrimidic endonuclease; MBD4, methyl binding domain protein 4; MYH, MutY homolog; NEIL1, Nei-like 1; NTH1, endonuclease three homolog 1; OGG1, 8-oxoguanine DNA glycosylase; UNG, uracil DNA glycosylase; THF, tetrahydrofuran; ODN, oligonucleotide.

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evidence, was brought by the demonstration that oxidative stress triggered APE1 accumulation in the nucleus (Mitra et al., 2007).

Recently, we have shown using a multiplexed excision/synthesis repair assay on damaged plasmid biochip that aging was associated with a drastic decrease of global excision/synthesis capacity of human fibroblast nuclear extracts toward lesions repaired by BER and NER (photoproducts, 8oxoG, and AP sites). Decrease was marked, however not significant, toward alkylated bases and Glycols (Sauvaigo et al., 2010). This global effect was mainly attributed to decline in polymerase functions, although an alteration of upstream repair regulation pathways should also be considered possible.

In the present study, in order to bring complementary information on the effect of aging on repair activities, we used a multiplexed oligonucleotide (ODN) cleavage assay on support to monitor cleavage efficiency of glycosylases contained in the fibroblast extracts toward several emblematic base lesions. Hence, we focused on initial steps of BER, that is to say the combined action of glycosylase and AP endonuclease or AP endonuclease alone, leading to cleavage of the altered bases.

As solar irradiation is responsible for the generation of oxidative DNA damage and photoproducts (mainly cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts (64-PP)) through the formation of ROS and by direct absorption by the DNA, respectively (Moriwaki and Takahashi, 2008), we also investigated the consequences of chronic sun-exposure by using fibroblasts grown from biopsies taken from photo-protected and photo-exposed areas of the same donors.

2. Materials and methods

2.1. Subjects

Twenty-nine healthy Caucasian females were recruited by Dermscan (Lyon, France). Biopsy removal was performed in accordance with ethical procedures (Helsinki Guidelines) after approval by a Medical Ethics Committee (CCPPRB). Three groups were constituted: group 1: 20–33 years old (mean age = 25 years; 9 subjects); group 2: 40–50 years old (mean age = 46 years; 9 subjects); group 3: 61–68 years old (mean age = 65 years; 14 subjects). All subjects were non-smokers, had phototype II or III skin, declared no excessive exposure to sun or UVA, no cutaneous pathology, and no medical treatment.

2.2. Cell culture

Two 3 mm punches were taken for each volunteer: one on dorsal forearm (photo-exposed area (PE)) and one on upper inner arm (photo-protected area (PP)). The fibroblast cultures were established by outgrowth of the punches previously cut into small pieces. The cells were grown in M199 medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% FCS and containing 100 U per mL penicillin and 100 μg per mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Cells were harvested during the exponential phase of growth at 70% confluence and stored frozen in liquid nitrogen in culture medium containing 10% DMSO. All the cells were cryopreserved at passage 5 ($3–7 \times 10^6$ cells per pellets). Two PP biopsies from age group 3 were lost during fibroblasts culture.

2.3. Cell nuclear extracts

Nuclear extracts were prepared as already described (Millau et al., 2008). Rapidly thawed cells were washed twice in ice-cold PBS, suspended in 1 mL of ice-cold buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.01% Triton X-100, 0.5 mM DTT, 0.5 mM PMSF) and left on ice for 20 min. Cytoplasm membrane lysis was completed by vortexing the tube for 30 s. Lysis completion was controlled by trypan blue exclusion and nuclei were recovered by centrifugation 5 min at 5000 rpm at 4 °C. They were then suspended in 25 μL of ice-cold buffer B (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 400 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, antiproteases (Complete-mini, Roche, Meylan, France) and 0.5 mM PMSF). Nuclear membranes were lysed for 20 min on ice, and two cycles of freezing–thawing at −80 °C and 4 °C, respectively. The extracts were cleared by centrifugation for 10 min at 13,000 rpm at 4 °C. The supernatant was recovered and stored frozen in 10 μL aliquots at −80 °C. Protein content was determined using the BCA kit (Interchim, Montluçon, France). Typical protein content was 1 mg/mL.

2.4. Preparation of lesion-ODN biochip

We used the concept of multiplexed zip-code ODN array, already described, to functionalize streptavidin glass slides with series of lesion-containing ODNs (Sauvaigo et al., 2004; Guerniou et al., 2005), except several material changes that are detailed below. Optimized concentration of biotinylated support-ODNs (1–1.5 μM in PBS) were printed in duplicate in a 24-well format on streptavidin glass slides (Xantec Bioanalytics GmbH, Germany). The slides were then set into ArrayIt® microplate hardware to demarcate the wells. Duplexes pre-formed by the specific hybridization of one lesion-ODN Cy3-labeled and one long-ODN were mixed and hybridization was conducted in each well for 1 h at 37 °C in a total volume of 80 μL (final duplex concentration 0.5 μM in PBS containing 0.2 M NaCl). Slides were then rinsed 3× 5 min with 80 μL of excision buffer (10 mM Hepes/KOH pH 7.8, 80 mM KCl, 1 mM EGTA, 0.1 mM $ZnCl_2$, 1 mM DTT, 0.5 mg/mL BSA). The sequences of the lesion-ODNs are listed in Table 1.

Table 1
Sequences of the modified oligonucleotides used. All the lesion-ODNs were labelled by Cy3 at their 3′-end. The lesions were situated 6–9 bases from the 3′-end of the ODNs and as far as possible, positioned within an identical sequence context.

Cleaved lesions/ bases-paired bases	Lesion-ODNs (sequence 5′ → 3′) **X** = modified base; in bold: cleaved base
Tg–A	CAC TTC GGA TCG TGA C**X**G ATC T
THF–A	CAC TTC GGA TCG TGA C**X**G ATC T
U–G	CAC TTC GGA TCG TGA **X**TG ATC T
U–A	CAC TTC GGA TCG TGA **X**TG ATC T
8oxoG–C	CAC TTC GGA TCG T**X**A CTG ATC T
A–8oxoG	CAC TTC GGA TCG TG**A** CTG ATC T
T–CpG	CAC TTC GGA TCG TGA C**T**T ATC T
Hx–T	CAC TTC GGA TCG TG**X** CTG ATC T
Control-ODN	CAC TTC GGA TCG TGA CTT ATC T

Each well contained a control-ODN, and 8 lesion-containing ODNs, all in duplicate: 8oxoG paired with C, A paired with 8oxoG, T mispaired with G in a CpG context, hypoxanthine in front of a T, Tg paired with A, tetrahydrofuran (THF), as AP site substrate equivalent, paired with A, U paired with G on the one hand and with A on the other hand.

It must be noted that, as far as possible, all lesions were placed in the same sequence context to limit possible differences in cleavage efficiency due to differences in the nature of adjacent bases.

2.5. Excision reaction

Typically, on each 24-well slide, 6 control wells that contained excision buffer alone and 18 excision reaction wells were distributed. Nuclear extracts (20 μg/mL in 80 μL of excision buffer) were added to the wells and incubated at 30 °C for 1 h. Each extract was tested in duplicate. After completion of the excision reaction, slides were rinsed 3× 5 min in PBS containing 0.2 M NaCl and 0.1% Tween 20. Excess buffer was removed by centrifugation, and the slides were dried before quantification of the residual fluorescence at 532 nm wavelength using a Genepix 4200A scanner (Axon Instrument, Molecular Devices, Sunnyvale, CA, USA). Total spot fluorescence intensity was calculated using the Genepix Pro 5.1 software (Axon Instrument). Results were normalized using the NormalizeIt software as described (Millau et al., 2008).

For each slide, normalized fluorescence level of control wells (repair buffer alone) was taken as reference and fluorescence level of each lesion-ODN of control well was set up to 100. Excision rate at the level of each lesion was then calculated as a percentage of the fluorescence obtained in control wells. In addition, each well contained a control-ODN, without any lesion, which served to check for any unspecific degradation activity. Typically degradation at the level of this control-ODN was between 0 and 10%. For calculation of final lesion-ODN cleavage percentage, a correcting factor was applied that took into account the possible control-ODN degradation. Consequently this final lesion-ODN cleavage percentage was 100 × (1 − percentage of fluorescence of lesion-ODN/percentage of fluorescence of control-ODN).

2.6. Statistical analysis

ANOVA was employed to analyze effect of aging (according to age group status) and photo-exposure on repair activities. All statistics were run using SigmaStat® V3.1 software.

3. Results

3.1. Cleavage efficiency

Examination of the average cleavage percentage for each lesion revealed that 3 groups of lesion substrates, classified according to

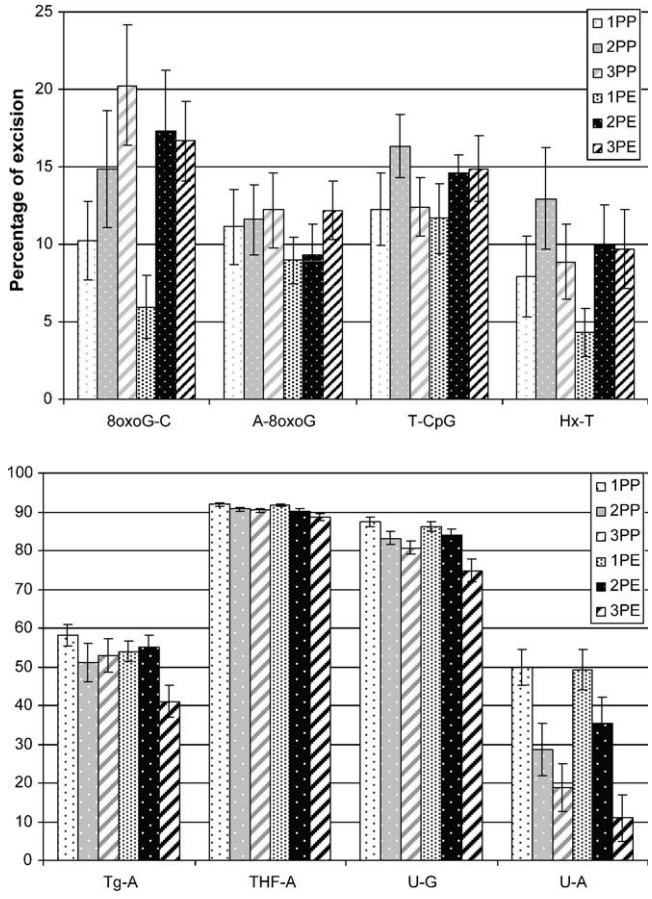

Fig. 1. Efficiency of cleavage of the different lesions using the multiplexed ODN cleavage assay. The data are clustered by age groups (1–3) and with respect to their exposure status (PP and PE). (A) Excision activities toward 8oxoG–C, A–8oxoG, T–CpG and Hx–T and (B) excision activities toward Tg–A, THF–A, U–G and U–A, expressed as percentage of cleavage of total fluorescence intensity with respect to the initial total fluorescence intensity. Data are reported as means and standard errors of the means. According to bifactorial ANOVA, chronic photo-exposure had no significant effect on repair activities whereas age was significantly associated with changes in the repair of 8oxoG–C, Tg–A, THF–A, U–G, and U–A (see Section 3 for significance).

cleavage efficiency, could be distinguished: one associated with very high cleavage efficiency (90% and 80% for THF and U:G, respectively), one with intermediate cleavage efficiency (30% for U paired with A and 50% for Tg). The third group included 8oxoG, A paired with 8oxoG, T at CpG site and Hx facing T for which cleavage efficiency was typically less than that 15%.

3.2. Effect of donor age and of photo-exposure on cleavage efficiency of the different lesions

Bifactorial ANOVA was performed to evaluate the effect of age (by age group) and of photo-exposure on the DNA repair activities. The analysis did not show any significant effect of chronic photo-exposure on the cleavage efficiency of the different substrates.

Monofactorial ANOVA was thus applied to determine the effect of age using the whole set of data. Results expressed as averaged cleavage percentage for each lesion as a function of age group and according to the photo-exposure status are displayed in Fig. 1. The analysis revealed that aging had a significant effect on cleavage efficacy of THF:A ($p = 0.006$), U:G mispair ($p < 0.001$), U:A base pair ($p < 0.001$), Tg:A ($p = 0.043$) and 8oxoG:C ($p = 0.01$) whereas no clear effect was demonstrated for the other lesions. Although

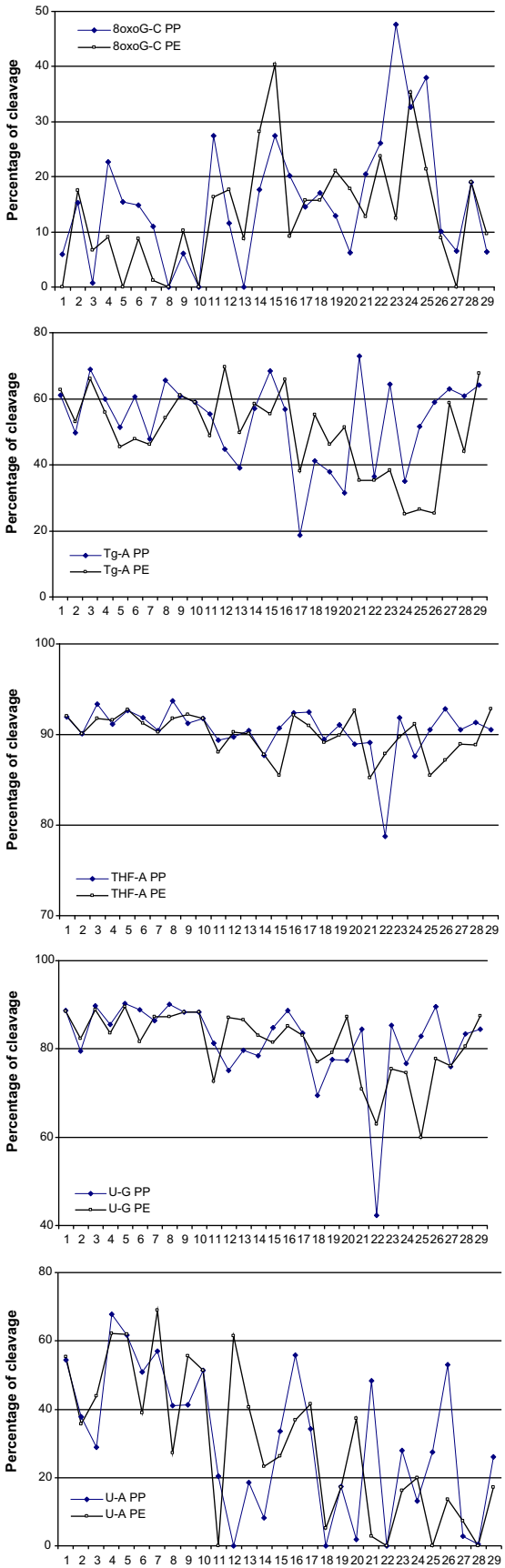

Fig. 2. Representation of the efficiency of cleavage of 8oxoG–C, Tg–A, THF–A, U–G and U–A by the extracts, using the normalized PP data positioned one curve (solid line) and the normalized PE paired data positioned on the other curve (dotted line). The individuals are classified by increasing ages (age group 1: samples 1–9; age group 2: samples 10–17; age group 3: samples 18–29).

the decrease in cleavage efficiency of THF was weak, it was significant between age group 1 and age group 3 ($p = 0.0015$). Cleavage of U facing A was about 2 times lower than cleavage of U:G in age group 1. Strikingly cleavage efficiency of U:A decreased very fast and significantly between each age group and reached a very low level in age group 3 (<20%) whereas cleavage decrease of U:G although significant between age groups 2 and 3 remained around 75% (see Fig. 1 for significance).

Unexpectedly, whereas the age was inversely correlated with cleavage efficiency for THF, U:G, U:A, a positive correlation was observed in the case of 8oxoG, revealing an up-regulation of the related excision rates with aging. The latter lesion was the sole one for which this observation was made.

All repair reactions were conducted simultaneously on the different substrates in the same wells with the same specimen extracts and thus bias in the experimental procedure cannot be responsible for the measured effect.

As statistics did not show any significant effect of photo-exposure on the repair activities, the two points of the same individuals could be considered as duplicates. In order to better visualize the results and in particular to evaluate the inter-individual and the intra-individual variability, data (percentage of cleavage of the lesions for all samples classified according of the donors age) relative to PP and PE samples for each individual were also displayed in Fig. 2 as superposed curves for some of the lesions (8oxoG–C, Tg–A, THF–A, U–G and U–A). It can be observed that the inter-individual and intra-individual variability, whatever the exposure status, was rather small in age group 1 whereas data were clearly more dispersed in age groups 2 and 3. In addition, low cleavage efficiency (such as for 8oxoG and U–A) was also associated with more scattered data. At this stage we cannot formally associate the variability observed between the duplicates to experimental error. A possible heterogeneous impact of the chronic exposure should also be considered.

Changes in the repair capacities occurred around the age of forty in the female population investigated here. Hence, heterogeneity in the DNA repair response seems to be a hallmark of aging and might reflect differences in the genetic susceptibility of the individuals as well as differences in their exposure to genotoxics during life time.

4. Discussion

Our study addresses effect of aging on excision capacity of primary human fibroblast extracts using a multiplexed ODN cleavage assay. To our knowledge, this is the first comparative assay allowing such complete hierarchical classification of relative cleavage efficiency of different base/sugar lesions by human extracts and the application to study the consequences of aging. Observation of BER phenotypes revealed a wide range of efficacy between the different excision activities. In accordance with data published by others using natural AP sites, we found that THF, the synthetic AP site, was the most easily repaired lesion compared to all the other lesions (Cappelli et al., 2001; Visnes et al., 2008). Except for U and Tg which exhibited intermediate cleavage rates, other lesions or mispairs present on the support were repaired with poor efficiency (Fig. 1).

APE1 is abundant in human cells and accounts for nearly all of the abasic site cleavage activity observed in cellular extracts (Meihua and Kelley, 2004). APE1 is a key protein of BER, not only catalyzing the cleavage of abasic sites but also, as a second player, involved in cleavage of most oxidized and alkylated bases. APE1 facilitates turnover of UNG, OGG1, TDG, MYH and NTH1. Hence a large role as coordinating BER protein is attributed to APE1 (Izumi et al., 2003). Despite the fact that efficiency of THF was about 90%, in the 3 considered age groups, slight but significant decrease with age was put in evident. The excision rate at the level of the different lesions present on the support reflected the combined action of glycosylase and APE1. Thus, the slight decrease in APE1 activity could limit its availability either for displacement of glycosylases or for cleavage of AP sites resulting from glycosylase action. As a consequence the slight decrease of APE1 activity could be partly responsible for the repair decline observed at the level of several base lesions *in vitro*. Indeed this could also apply *in vivo* with drastic consequences in terms of mutation risk.

Whereas repair of U:G, although declining with age, remained quite efficient (above 75% cleavage in the elderly), drastic effect of aging was observed on repair of U:A base pair. Strikingly, in several donors belonging to the elderly group it was almost undetectable (data not shown). Misincorporation of uracil opposite A is the major contributor to the presence of U in DNA (Visnes et al., 2008) whereas U placed opposite G mimics cytosine deamination. Although several human glycosylases possess the ability to cleave U from DNA, UNG2 was demonstrated to be the sole one glycosylase responsible for initiation of repair of all U:A and of the largest fraction of U:G by nuclear extracts (Krokan et al., 2001). TDG and SMUG1 could contribute to initiation step and complement UNG2 in case of poor expression but only for U:G pairs (Visnes et al., 2008). Our results clearly showed that in a competition situation U:G cleavage is favoured. Consistent with data from Visnes et al, we can speculate that no backup glycosylase exists for repair of U:A. Despite the fact that U:G is mutagenic and U:A is not, biological consequences of impairment of repair of the latter base pair with aging would be important to elucidate.

8OxoG is the most common purine lesion formed upon oxidative attack of DNA and exhibits high mutagenic potential (Grollman and Moriya, 1993). In accordance with earlier studies, we found that excision rate of 8oxoG was weak (Cappelli et al., 2001; Eiberger et al., 2008). In human repair of 8oxoG is initiated by OGG1 which possesses an intrinsic AP lyase activity. Alone, this enzyme has low turnover and is inhibited by its own product. However in the presence of APE1, specific activity of OGG1 is increased about five-fold (Hill et al., 2001). Among all the excision activities tested, cleavage of 8oxoG is the sole for which a significant increase with aging was put in evident. Considering our finding on alteration of APE1 activity with aging, this enhanced 8oxoG cleavage cannot be attributed to the action of APE1. Despite recent data showing increased 8oxoG incision rates by human lymphocyte extracts with aging (Humphreys et al., 2007), several studies, mostly conducted in mouse, agreed on a decline of nuclear OGG1 activity with age (Xu et al., 2008). Importantly, it appears that a different regulation could take place in mitochondria where the 8oxoG incision activity increased with age (de Souza-Pinto et al., 2001; Xu et al., 2008), although this feature seems to be tissue-specific (Gredilla et al., 2010). APE1 and OGG1 are redox sensitive enzymes and it has been shown recently that oxidative stress impaired cleavage activity of OGG1 (Eiberger et al., 2008) whereas it induced APE1 de novo synthesis and translocation from the cytosol to the nucleus (Mitra et al., 2007). So apparently, oxidative stress, intimately associated with aging, has opposite effects on these two coordinated enzymes for 8oxoG processing in the nucleus. As a matter of fact, the results obtained in our experimental conditions demonstrated a fundamental difference between OGG1/APE1 and the other glycosylase/APE1 activities.

Thymine glycol is the main product resulting from oxidation of pyrimidine bases and possesses strong replication blocking properties (Dianov et al., 2000). Repair of this substrate in mammalian cells is mainly attributed to hNTH1 although NEIL1 which acts mainly in transcriptionally active regions could also be

involved (Hegde et al., 2008). In our study cleavage of Tg was moderately but significantly affected by aging.

For other lesions which as 8oxoG exhibited low excision rates, no evolution with age was detected. This concerned repair of A facing 8oxoG by MYH, hypoxanthine by AAG, and repair of T in a CpG context presumably by MBD4.

In a recent paper (Sauvaigo et al., 2010), using the same set of cell extracts, we have shown that a marked excision/synthesis decrease was associated with aging for lesions repaired either by BER or by NER (data not shown). In particular, repair of 8oxoG declined between age groups 1 and 2, and then remained stable in group 3 (data not shown). Using this latter assay, it was not possible to determine the contribution of the different steps involved in repair (excision/cleavage/DNA synthesis) to the observed decline with aging. Data from the literature suggested that this decline could be, at least partly, attributed to impaired polymerase activity (Raji et al., 2002; Takahashi et al., 2004). We can thus speculate that the increase we observed here was masked by the drop in the polymerase activity in the former assay. These apparently contradictory results underline the critical requirement for a clear distinction between the different steps composing the whole repair process.

Results obtained in the present study complete our investigation on the consequences of aging on DNA repair mechanisms and bring additional information focused on the excision step of lesions repaired by glycosylases/AP endonuclease. Considering the format of our assay and because of the size of the ODNs and the position of the base lesions (6–9 bases from the 3′ end of the ODNs; Table 1), we assume that only small protein complexes can get fixed onto the base lesions. We know from numerous *in vitro* studies that indeed glycosylases can load, operate base flipping and cleave the damaged base on such small fragments (review in Ide, 2001). However, we cannot exclude the possibility that the catalytical rate of certain enzymes we measure here might not be optimum because some "helper" proteins cannot assemble on such small fragments or in our experimental conditions (low protein concentration).

In conclusion, we demonstrated that a decline of the initial step of BER due to aging was a general feature of the most abundant nuclear BER proteins. We attributed this decline to impaired excision activities. However, it would also be interesting to investigate in details if certain helicase/nuclease activities could contribute to this decline by a destabilization/displacement mechanism of the duplex-ODNs present on the support.

Moreover, marked difference between U:A and U:G repair was observed which underlined the critical importance of the nature of the base pair investigated. On an other hand, the drastic decline of U:A repair suggested that this measure could be a good candidate as an aging marker of skin cells.

Understanding how 8oxoG repair is regulated seems particularly tricky. Considering the possible existence of backup repair systems that could be affected by aging and that do not operate in the experimental conditions of the ODN cleavage assay on support, it would be speculative to conclude that the increase we observed reflected an *in vivo* increased ability of cells to repair 8oxoG. As a matter of fact, results of our previous study using the same cell extracts revealed a decline of global excision/synthesis repair of 8oxoG with aging (Sauvaigo et al., 2010). However, the present data suggest a specific susceptibility to aging of BER pathway in charge of 8oxoG.

Finally, our global comprehensive tool provides an easy strategy to identify critical steps affected by aging that would require extensive biochemical investigations.

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