

Age-related and tissue-specific accumulation of oxidative DNA base damage in 7,8-dihydro-8-oxoguanine-DNA glycosylase (Ogg1) deficient mice

Marcel Osterod, Stephan Hollenbach, Jan G.Hengstler¹, Deborah E.Barnes², Tomas Lindahl² and Bernd Epe³

Institute of Pharmacy and ¹Institute of Toxicology, University of Mainz, D-55099 Mainz, Germany and ²Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK

³To whom correspondence should be addressed
Email: epe@mail.uni-mainz.de

Mutations that influence the repair of oxidative DNA modifications are expected to increase the steady-state (background) levels of these modifications and thus create a mutator phenotype that predisposes to malignant transformation. We have analysed the steady-state levels and repair kinetics of oxidative DNA modifications in cells of homozygous *ogg1*^{-/-} null mice, which are deficient in Ogg1 protein, a DNA repair glycosylase that removes the miscoding base 8-hydroxyguanine (8-oxoG) from the genome. Oxidative purine modifications including 8-oxoG were quantified by means of an alkaline elution assay in combination with Fpg protein, the bacterial functional analogue of Ogg1 protein. In primary hepatocytes of adult *ogg1*^{-/-} mice aged 9–12 months, the steady-state level of the lesions was 2.8-fold higher than in wild-type control mice. In contrast, no difference between *ogg1*^{-/-} and wild-type mice was observed in splenocytes, spermatocytes and kidney cells. In hepatocytes of *ogg1*^{-/-} mice, but not in wild-type controls, the steady-state levels increased continuously over the whole lifespan. No significant accumulation of the oxidative base modifications was observed in *ogg1*^{-/-} fibroblasts in culture when they were kept confluent for 8 days. Both in confluent and proliferating *ogg1*^{-/-} fibroblasts, the global repair of additional oxidative base modifications induced by photosensitization was 4-fold slower than in wild-type cells. The results suggest that the consequences of an Ogg1 defect are restricted to slowly proliferating tissues with high oxygen metabolism such as liver, because of a back-up mechanism for the repair of 8-oxoG residues that is independent of transcription and replication.

Introduction

There is much evidence that reactive oxygen species (ROS) generated during normal cellular oxygen metabolism are an important source of endogenous DNA damage in all cell types and that the resulting oxidative DNA modifications contribute to the spontaneous mutation rates. Therefore, oxidative DNA damage has been implicated in carcinogenesis, the ageing process and several age-related degenerative diseases (1–4).

The generation of oxidative DNA damage is counteracted in all species by specific repair mechanisms (5–7). One of the

key repair enzymes in eukaryotic cells is Ogg1 protein, a DNA glycosylase that removes 7,8-dihydro-8-oxoguanine (8-oxoG) and other oxidative guanine modifications from the nuclear and mitochondrial DNA (8,9). Ogg1 is a functional, but not structural, analogue of the bacterial Fpg protein. Ogg1 deficiency in yeast, as well as Fpg deficiency in bacteria, results in a spontaneous mutator phenotype (10,11). GC→TA transversions, which are the characteristic type of mutation caused by 8-oxoG due to its ability to pair with adenine, are increased specifically. Recently, 2–3-fold elevated spontaneous mutation rates were also detected in the liver of homozygous *ogg1*^{-/-} null mice (12,13) and all additional mutations appeared to be GC→TA transversions. The background level of 8-oxoG in the chromosomal DNA of untreated animals, which probably reflects the balance between the continuous generation and removal of this modification (steady-state level), was significantly higher in the liver of *ogg1*^{-/-} mice than in that of wild-type animals. Moreover, the level of 8-oxoG in the mutant mouse liver at 14 weeks of age was nearly 2-fold higher than at 9 weeks of age, which suggests an accumulation rate of 12 000 modifications per diploid cell per week (13).

To further characterize the consequences of an Ogg1 deficiency, we have analysed the steady-state levels of oxidative base modifications sensitive to Fpg protein in various organs and along the lifespan of *ogg1* knockout mice. The results indicate that the accumulation of oxidative DNA modifications is tissue-specific, i.e. it is much more pronounced in hepatocytes than in several other cell types, most probably due to the high oxygen metabolism in the liver. An age-dependent increase of oxidative damage in *ogg1*^{-/-} mice indicates that either oxidative stress increases with age or the relevant cellular defence mechanisms become less efficient.

Materials and methods

Mice, cell lines, repair enzymes and chemicals

The generation of *ogg1*^{-/-} null mice has been described previously (12). Spontaneously immortalized embryonic fibroblasts were established from the *ogg1*^{-/-} and control mice (12) and cultured in 3 parts DMEM (high glucose) medium plus 1 part HAM's F12 medium, with 10% fetal calf serum in the presence of 100 U/ml penicillin and 100 µg/ml streptomycin. Formamidopyrimidine-DNA glycosylase (Fpg protein) (14) from *Escherichia coli* was kindly provided by S.Boiteux (CEA, Fontenay-aux-Roses, France).

[R]-1-[(10-Chloro-4-oxo-3-phenyl-4H-benzo[*a*]quinolizin-1-yl)-carbonyl]-2-pyrrolidinemethanol (Ro19-8022) was a gift from Hoffmann-LaRoche AG (Basel, Switzerland).

*Preparation of hepatocytes, splenocytes and kidney and testis cells from *ogg1*^{-/-} and wild-type mice*

Hepatocytes from *ogg1*^{-/-} and control mice were isolated by a modified two-step collagenase perfusion technique as previously described (15). Briefly, mice were anaesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg), the liver was perfused *in situ* via the vena portae for 15 min with an EGTA-containing buffer at 37°C. The perfusion was continued with a collagenase buffer for 30 min. All buffers were carbogen-equilibrated before and during preparation, the flow did not exceed ~10 ml/min. After perfusion, the liver was removed and the hepatocytes carefully dissociated in suspension buffer, the suspension filtered through gauze (100 µm pore size). Cells were pelleted by centrifugation (10 min at 50 g) and resuspended in fresh suspension buffer (15) three times. The viability of the hepatocytes was determined by

Abbreviations: Fpg protein, formamidopyrimidine-DNA glycosylase; 8-oxoG, 7,8-dihydro-8-oxoguanine; Ro19-8022, [R]-1-[(10-Chloro-4-oxo-3-phenyl-4H-benzo[*a*]quinolizin-1-yl)-carbonyl]-2-pyrrolidinemethanol; ROS, reactive oxygen species.

trypan blue (4 g/l) exclusion and ranged between 30 and 80%. The hepatocytes were subjected to analysis by alkaline elution (see below) within 1 h.

For the isolation of splenocytes, the spleen was cut into small pieces with surgical scissors in 10 ml trypsin/EDTA buffer (0.5 g/l trypsin, 0.2 g/l EDTA, 140 mM NaCl, 3 mM KCl, 8 mM NaH₂PO₄, 1.5 mM KH₂PO₄, pH 7.4) and incubated for 10 min at 37°C with gentle shaking. The reaction was stopped by the addition of 40 ml suspension buffer. Cells were pelleted by centrifugation (10 min at 150 g) and resuspended in an erythrocyte lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA, pH 7.4) for 5 min on ice. Thereafter, the cells were pelleted again and resuspended in fresh suspension buffer. The viability of the splenocytes as determined by trypan blue exclusion ranged between 65 and 85%.

Kidney cells were prepared in a similar way, except that the treatment with trypsin/EDTA was replaced by an incubation in collagenase buffer for 90 min at 37°C. Cells could be cryopreserved in suspension buffer containing 10% DMSO using a slow freezing protocol (15) and stored in liquid nitrogen.

Testes and seminal ducts of male mice (aged 20.5–26.5 months) were cut into pieces and gently shaken in pre-chilled suspension buffer for 10 min. The suspension was pelleted by centrifugation at 50 g for 10 min and left at room temperature to allow vital spermatozoa to swim into the supernatant. After 30 min, the supernatant (containing the spermatozoa) was removed from the pellet and the cells were counted in a Neubauer chamber. The percentage of motile spermatozoa in the supernatant was 80–90%.

Quantification of Fpg-sensitive modifications in DNA by the alkaline elution technique

The alkaline elution protocol followed the method of Kohn *et al.* (16) with modifications (17,18). The number of cells applied to each filter was 5×10^5 in the case of the hepatocytes and 1×10^6 in all other cases to compensate for the higher DNA content (polyploidy) of the hepatocytes. The sum of modifications sensitive to Fpg protein and single-strand breaks was determined following incubation of the cellular DNA for 60 min at 37°C with 1 µg/ml Fpg protein immediately after cell lysis. Under these conditions, the incision by the enzyme at sensitive DNA modifications has been shown to be completed (18). The numbers of modifications incised by Fpg protein were obtained by subtraction of the number of single-strand breaks observed in experiments without the enzyme. Elution curves obtained with γ -irradiated cells were used for calibration, assuming that 6 Gy generate 1 single-strand break per 10^6 bp (16). When induced modifications (rather than background levels) were to be quantified (repair assays), the slopes observed with untreated control cells (steady-state levels) were subtracted.

Determination of repair rates in cultured fibroblasts

To induce additional oxidative base damage for the determination of repair rates, spontaneously immortalized fibroblasts (10^6 cells/ml) were exposed to the photosensitizer Ro19-8022 (0.05 µM) in the presence of visible light from a 1000 W halogen lamp (Philips PF811) at a distance of 38 cm in Ca²⁺ and Mg²⁺-free PBS (140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.4) at 0°C for 10 min, corresponding to 14 kJ/m² between 400 and 500 nm. Treatment results in the induction of 0.5 Fpg-sensitive base modifications per 10^6 bp (~60% of which were identified as 8-oxoG) and relatively few other DNA modifications and does not reduce the viability of the cells (19). To remove the damaging agents, the treated cells were washed twice with Ca²⁺ and Mg²⁺-free PBS. Subsequently, the cells were resuspended in complete medium, incubated for various times under culture conditions at 37°C and analysed for unrepaired DNA modifications, as described above.

Results

Accumulation of oxidative DNA modifications in *ogg1*^{-/-} mice is tissue specific

An alkaline elution technique was used to quantify the number of modifications sensitive to Fpg protein in the chromosomal DNA of cells isolated from liver, spleen, kidney and testis of wild-type versus homozygous *ogg1*^{-/-} null mice. Knockout and control mice were aged between 9.5 and 11.3 months (between 20.5 and 26.5 months in the case of spermatozoa).

The results (Figure 1) indicate that the steady-state level of Fpg-sensitive modifications in the hepatocytes of *ogg1*^{-/-} mice was 2.8-fold higher than in wild-type mice. In contrast, no significant difference was observed for the other tissues.

Accumulation of oxidative DNA modifications in *ogg1*^{-/-} mice is age-dependent

In Figure 2, the levels of Fpg-sensitive modifications determined in the hepatocytes of wild-type and *ogg1*^{-/-} mice is

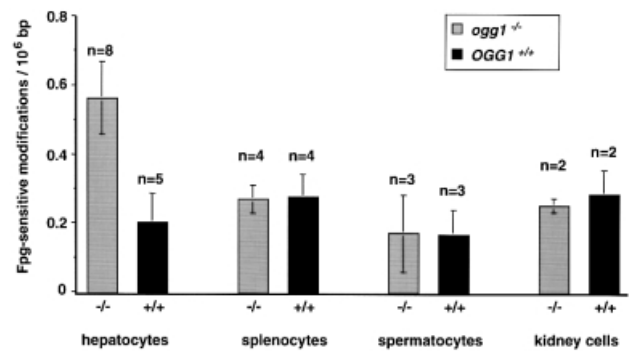


Fig. 1. Steady-state (background) levels of Fpg-sensitive DNA modifications in various cell types isolated from *ogg1*^{-/-} mice and wild-type controls (aged 9.5–11.3 months; 20.5–26.5 months in the case of spermatozoa). Data are means ± SD; the number of animals analysed is indicated above the columns.

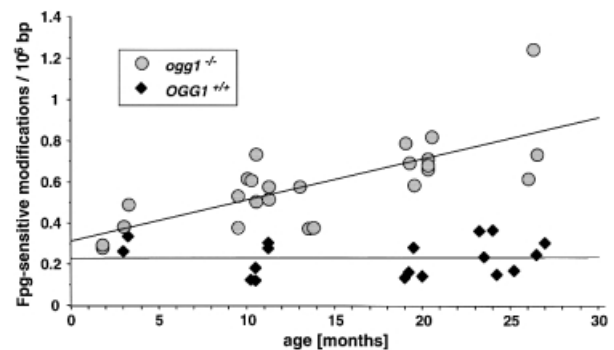


Fig. 2. Age-dependence of the steady-state levels of Fpg-sensitive DNA modifications in primary hepatocytes isolated from *ogg1*^{-/-} mice (●) and wild-type controls (◆) aged from 2 to 27 months.

plotted against the age of the animals. The data indicate a highly significant age-dependent increase of the level of DNA modifications for the knockout mice over their whole lifespan ($P < 0.0001$). The accumulation rate is apparently constant (~0.01 Fpg-sensitive modification per 10^6 bp per week) and much lower than that calculated on the basis of the two early time points reported by Minowa *et al.* (13). No significant increase of the steady-state levels was observed in the hepatocytes of wild-type mice under the same conditions (Figure 2) and in the splenocytes of the knockout mice between 10 and 20 months (data not shown).

No correlation was observed between the steady-state levels of Fpg-sensitive modifications and the viability of the isolated hepatocytes determined by trypan blue exclusion (Figure 3, upper panel), nor was the viability of the hepatocytes significantly correlated with age (data not shown). This indicates that neither the age-dependent increase nor the interindividual variation of the steady-state levels of Fpg-sensitive modifications is an artefact caused by variable cellular damage during the isolation procedure. In contrast, the background level of DNA strand breaks was significantly increased in cells with reduced viability and was independent of the *ogg1* genotype (Figure 3, lower panel).

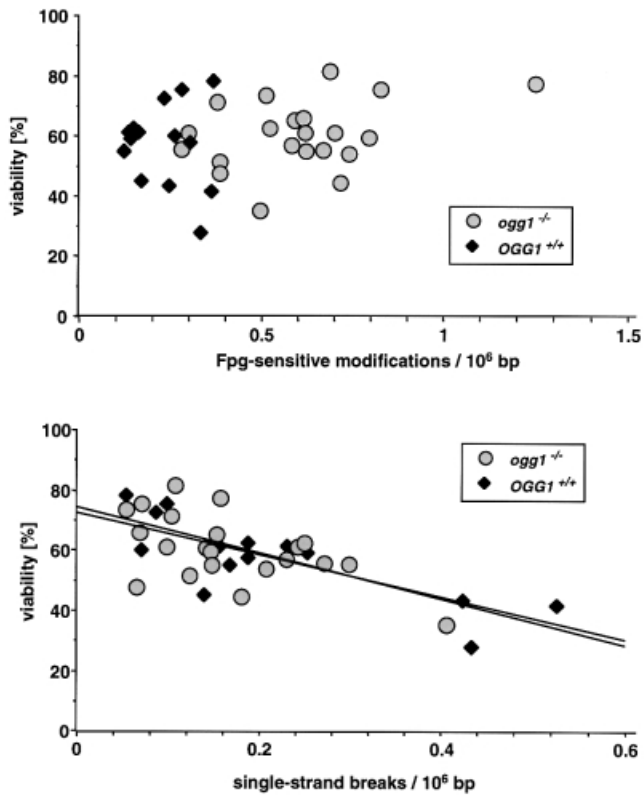


Fig. 3. Correlation of the levels of Fpg-sensitive modifications (upper panel) and single-strand breaks (lower panel) in primary hepatocytes isolated from *ogg1*^{-/-} mice (○) and wild-type controls (◆) with the viability of the cells determined by trypan blue exclusion.

*Cell proliferation is not relevant for the accumulation of oxidative DNA modifications in *ogg1*^{-/-} cells and has no influence on the repair rates*

A putative explanation for the accumulation of oxidative DNA modifications in *ogg1*^{-/-} mice being much higher in liver than in other tissues analysed is that the cell proliferation rate is much lower in liver. Cell proliferation could reduce the steady-state levels of oxidative DNA modifications in two ways: (i) each cell division dilutes the level of damage by a factor of two if there is no or little incorporation of modified bases during DNA replication; (ii) the DNA repair rate might be higher in replicating cells than in resting cells.

In order to analyse the influence of cell proliferation, we (i) determined the steady-state levels of Fpg-sensitive base modifications in proliferating and confluent immortalized embryonic fibroblasts derived from *ogg1*^{-/-} mice and (ii) compared the repair rates of additionally induced Fpg-sensitive base modifications in confluent and replicating *ogg1*^{-/-} fibroblasts.

There was no significant increase of the steady-state level of Fpg-sensitive DNA modifications when cultured *ogg1*^{-/-} fibroblasts reached confluence and were kept confluent for 8 days (data not shown). Therefore, the balance between the generation and removal of the oxidative base modifications is not instantly affected by DNA replication.

The repair kinetics in confluent and proliferating fibroblasts of additional Fpg-sensitive base modifications induced by exposure to the photosensitizer Ro19-8022 in the presence of light (19) is shown in Figure 4. Both for the *ogg1*^{-/-} and wild-

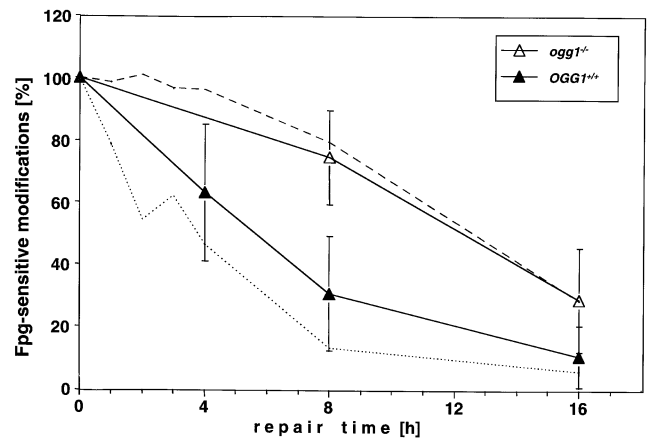


Fig. 4. Repair of Fpg-sensitive DNA modifications induced by 0.05 μ M Ro19-8022 plus visible light at 0°C in confluent immortalized embryonic fibroblasts from *ogg1*^{-/-} (Δ) and *OGG1*^{+/+} (\blacktriangle) mice. The numbers of lesions detected at various times after the treatment were corrected for the number of lesions in untreated control cells. Dashed lines (--- for *ogg1*^{-/-}; for *OGG1*^{+/+}) representing data for proliferating cells (corrected for the dilution of the induced DNA damage by cell proliferation) are shown for comparison (taken from ref. 12). The number of induced Fpg-sensitive modifications (100%) was $\sim 0.5/10^6$ bp. Data represent the means of three to five independent experiments (\pm SD).

type fibroblasts, similar repair rates were observed in dividing and non-dividing cells. Therefore, the previously described significant repair of Fpg-sensitive modifications in *ogg1*^{-/-} cells (12), which is ~ 4 -fold slower than in wild-type cells, does not depend on cell proliferation. A putative replication-coupled repair mechanism for 8-oxoG can be ruled out.

Discussion

The results demonstrate a highly tissue-specific accumulation of oxidative DNA base modifications in *ogg1*^{-/-} mice. The basal levels of Fpg-sensitive modifications, which include 8-oxoG and possibly some other oxidative purine modifications (14,20–22), were 2.8-fold elevated in liver, but normal in sperm, kidney and spleen (Figure 1). This finding provides an explanation for the previous observation that the mutation frequency in the *lacI* gene of *ogg1*^{-/-} *Big Blue* mice was 2–3-fold increased in the liver, but not in testis cells, in spite of the higher proliferation rate of the latter cell type, which generally favours mutagenesis (12).

The results further indicate that the tissue-specific accumulation of oxidative DNA damage in liver compared with other tissues cannot be explained by the lower level of cell turnover since (i) cell-cycle arrest alone does not change the level of oxidative DNA damage and (ii) the residual repair rate in *ogg1*^{-/-} fibroblasts is the same in proliferating and confluent cells (Figure 4). Therefore, it appears likely that a higher rate of generation of oxidative DNA damage rather than a reduced rate constant of removal is responsible for the selective accumulation in liver. The residual (back-up) repair activity in *ogg1*^{-/-} cells (Figure 4) probably is sufficient to keep the steady-state levels at a minimum in most tissues, at least in the absence of additional oxidative stress, but it is too low to prevent an increase of the steady-state level in the case of metabolically highly active cells such as hepatocytes.

The residual repair activity in *ogg1*^{-/-} cells ($t_{1/2} \approx 16$ h) suggests that a balance between endogenous generation and

removal of oxidative modifications (steady-state) is reached within days. Therefore, the age-dependent increase of the steady-state levels in the hepatocytes of *ogg1*^{-/-} mice (Figure 2) is an indication that either the cellular antioxidant defence system deteriorates with age or the endogenous oxidative stress increases gradually. Experimental support for both effects has been described and forms the basis of free radical theories of ageing (2,23,24).

The absence of a significant age-dependent accumulation of oxidative DNA damage in hepatocytes of wild-type mice (Figure 2) contrasts with a previously observed 2–3-fold increase of 8-oxoG in the liver of wild-type rats during their lifespan (25). An experimental difference that could explain the discordant findings is that the technique described here excludes the possibly highly oxidized DNA from disintegrated or apoptotic cells and from mitochondria.

The nature of the back-up repair system remains to be established. The recently observed OGG1-independent transcription-coupled repair of 8-oxoG (26) cannot fully explain that >50% of all Fpg-sensitive modifications induced in *ogg1*^{-/-} cells are repaired (Figure 4), even if it is assumed that the photosensitizer might damage transcribed regions of the genomic DNA more efficiently than non-transcribed DNA. A hypothetically related replication-coupled repair mechanism is excluded by the repair in confluent cells. Nucleotide excision repair cannot be ruled out, although the comparison of spontaneous mutation frequencies in yeast *ogg1* and *rad14* single and double mutants provided only partial support for such an assumption (27).

The back-up repair and the resulting absence of a significant accumulation of oxidative DNA damage in most tissues could explain why the incidence of cancer and other age-related diseases proposed to be associated with oxidative DNA damage is not clearly increased in *ogg1*^{-/-} mice. In the case of the liver, the low level of cell proliferation might prevent cancer development in the absence of a tumor promoting activity. This notion is supported by recent data indicating that a significant age-dependent accumulation of spontaneous point mutations (including GC→TA transversions) only takes place in proliferating tissue (28). However, the consequences of an *OGG1* defect may become apparent in combination with other mutations that affect either the generation or the removal of oxidative DNA damage. Indeed, mutations in the *OGG1* gene have been observed in some human tumors of the kidney, lung and other organs (29,30) and also in a mouse strain characterized by accelerated senescence (31). This would support the notion that *OGG1* is a 'caretaker' gene which—when mutated—under some conditions can facilitate tumor development by increasing spontaneous mutation rates (32). The analysis of double knockout mice and cells will allow further studies of the relevance of oxidative DNA damage and its repair.

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