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8-Oxoguanine DNA glycosylase (Ogg1) causes a transcriptional inactivation of damaged DNA in the absence of functional Cockayne syndrome B (Csb) protein

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ABSTRACT

We have analysed the effect of oxidative guanine lesions on the expression of a transfected reporter gene in mouse embryonic fibroblasts deficient in Cockayne syndrome B protein (Csb) and/or the 8-oxoguanine DNA glycosylase (Ogg1). We used a highly sensitive flow cytometry-based approach and quantitative real-time PCR to measure the changes in gene expression caused by the presence of oxidised guanine residues generated by photosensitisation in the vector DNA. In wild-type cells, small numbers (one or three) of oxidised guanines did not affect gene expression at short times after transfections, whereas progressive reduction of the transgene expression was observed at later time points. Although Ogg1 has a major contribution to the repair of oxidised guanine bases, its absence did not have a strong effect on the gene expression. In contrast, the lack of functional Csb protein caused a pronounced inactivation of the damaged reporter gene. Most strikingly, an additional Ogg1 deficiency significantly attenuated this effect. The results indicate that the processing of oxidative guanine modifications by Ogg1 can mediate host cell inactivation rather than reactivation of the damaged genes and that this effect is strongly enhanced in the absence of Csb.

1. Introduction

Oxidation of DNA bases by endogenously generated and environmental reactive oxygen species (ROS) leads to mutations that have been linked to cancer and aging (for a recent review, see Ref. [1]). Guanine is particularly prone to oxidation by ROS. 7,8-Dihydro-8-oxoguanine (8-oxoG; 8-oxoguanine) is one of the most frequent oxidation products of guanine and the most studied one because of its miscoding potential during replication and transcription [2,3]. Oxidised guanines are efficiently removed from chromosomal DNA by base excision repair initiated by the 8-oxoguanine DNA glycosylase Ogg1 [4–6] and, under certain conditions, by the DNA glycosylases/AP lyases NEIL1 and NEIL2 [7]. Cockayne syndrome B protein (Csb), which is a key factor required for the transcription-coupled repair (TCR), a subpathway of nucleotide-excision repair (NER), has been shown to participate in the repair of 8-oxoG [8,9] and to reduce the accumulation of the characteristic GC to TA mutations which arise in the absence of Ogg1 *in vivo* [10]. At the same time, there is little evidence of mechanistic relevance of NER to the repair of oxidative base lesions, including 8-oxoG, in mammalian cells.

As far as it is known, TCR is triggered by block of transcription at bulky DNA lesions [11]. 8-OxoG is not bulky, and its transcription-blocking potential is a matter of controversy. Elongating RNA polymerases bypass 8-oxoG with rather high efficiencies in reconstituted transcription systems [12–15], and transcription by RNA polymerase II can be further improved in presence of TFIIS, TFIIF, Elongin and Csb [13]. On the other hand, host cell reactivation studies of the plasmids damaged by photosensitisation revealed a decreased gene expression in presence of oxidative guanine lesions [16]. This was interpreted as a consequence of transcription block at the 8-oxoG [17], since the expression of the plasmids damaged with photosensitiser and those harbouring single synthetic 8-oxoG was improved by Csb [16,17] and Ogg1 [17]. However, in another study a single 8-oxoG residue within the transcribed strand caused no significant decrease of gene expression, and the deficiencies of Csb and Ogg1 had no significant effect [18].

To further study transcriptional toxicity of oxidative guanine lesions, we improved the sensitivity and reproducibility of the host cell reactivation assay using flow cytometry as a detection tool and measured the mRNA levels by real-time quantitative PCR. Our results indicate that the oxidative guanine modifications predispose to a host cell-mediated gene inactivation rather than reactivation, in marked contrast to the situation observed with UV-induced pyrimidine dimers. The observed decrease of gene

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expression appears to be accelerated by Ogg1 and counteracted by the presence of functional Csb.

2. Materials and methods

2.1. Plasmids

pEGFP-C3 and pDsRed-Monomer-N1 plasmids were from Clontech (Saint-Germain-en-Laye, France). We have further constructed a pEGFP-mODC-AZ plasmid that encodes for a destabilised green fluorescent protein [19] and pEGFP-mODC-ZA that has the same size and encodes for unaltered EGFP. Unlike the previously described pMAZ and pMZA plasmids (GeneBank accession numbers EU421131 and EU421132), the new plasmids cannot replicate in the cell lines used. A fragment of mouse *Odc* gene exon 10 (Mouse Genome Informatics; accession No. MGI:97402) was produced by PCR using *PfuTurbo*® DNA Polymerase (Stratagene, Amsterdam, The Netherlands) and the following primers: 5′-ACTCACTCATGAAGCAGATCCAGA-3′ and 5′-ACTAGCATCTACACATTGATCCTA-3′. The PCR product was blunt cloned into the ScaI site in the pEGFP-C3 plasmid. The resulting plasmids were sequenced, and the protein expression and lifetimes verified in transfected cells, as previously described [19].

2.2. Generation of damaged plasmid DNA

Covalently closed plasmids were damaged with light from a halogen lamp (Philips PF811) in the presence of 0.8 μM methylene blue (Sigma–Aldrich, Taufkirchen, Germany) or 50 μM Ro19-8022 (Roche, Basel, Switzerland), as described previously [20,21]. Average numbers of strand breaks (SSB) were calculated by the Poisson formula from the relative frequencies of covalently closed and nicked plasmid molecules determined after separation of the two forms by gel electrophoresis. Oxidised guanines and sites of base loss (AP sites) were quantified after full conversion into SSB by treatment with an excess of specific DNA repair endonucleases as previously described [20,21]. 70% of the Fpg-sensitive base modifications induced by Ro19-8022 plus light were identified as 8-oxoG [21]. UV photoproducts, mostly cyclobutane pyrimidine dimers (CPD), were generated by UVB as previously described [22]. Up to 3 CPD per plasmid molecule were quantified directly by incision with T4 endonuclease V. To generate 9 T4 endonuclease V-sensitive lesions per plasmid molecule, we assumed a linear dose–effect relationship and used a proportionally increased UV dose.

2.3. Cell lines and culture conditions

Spontaneously immortalised mouse embryo fibroblast (MEF) cell lines derived from *Ogg1*$^{-/-}$, *Csb*$^{m/m}$, and *Csb*$^{m/m}$/*Ogg1*$^{-/-}$ mice and corresponding wild-type MEFs (F11.1) were described previously [8]. MEF cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM sodium pyruvate, 100 U/mL penicillin, 100 mg/L streptomycin, and 15% foetal calf serum (all reagents from PAA Laboratories GmbH, Pasching, Austria). Human MRC5 foetal lung fibroblasts and HeLa cervical carcinoma cells were cultivated at 10% foetal calf serum. The cell cultures were maintained at low passage numbers and proven to be free of mycoplasma contamination. Repair rates of Fpg-sensitive oxidative base modifications were consistent with those published earlier [8]. Genetic identities of the MEF cell lines were confirmed by routine laboratory controls. In addition, wild-type and disrupted *Ogg1* and *Csb* alleles were detected using PCR analyses of total DNA that was isolated from the cell samples after the expression analyses.

2.4. Transfections

Exponentially growing cells were taken for transfections. HeLa and MRC5 cells were transfected in full medium in six-well plates (Nunc, Wiesbaden, Germany) with 800 ng of pEGFP-C3 plasmid with the help of Effectene® (Qiagen, Hilden, Germany). Plasmid and transfection reagent were removed after 4 h, and cells analysed at 24 h after transfection start. MEF cell lines were subjected to magnet-assisted transfection with MATra-A system (IBA, Göttingen, Germany). Cells were transfected for 20 min at the magnet plate and incubated for the indicated times after medium exchange. Co-transfections were done with equal amounts (1.5 μg) of the specified GFP-encoding plasmids (non-damaged or carrying the indicated numbers of DNA lesions) and non-damaged reference plasmid encoding for DsRed-Monomer. Double amounts of both plasmids were used in the experiments with UV-damaged plasmids, due to very low protein expression levels in *CSB*$^{m/m}$ cells. Green fluorescent protein (GFP) fluorescence was visualised by fluorescence microscopy and measured by flow cytometry (BD FACSCalibur™, Beckton Dickinson GmbH, Heidelberg, Germany) at 24 h after transfections, unless otherwise specified. For simultaneous mRNA and protein expression analyses, the cells were split 4 h post-transfection, and each portion was processed accordingly.

2.5. Protein expression analyses

Cells were fixed by formaldehyde prior to FACS analyses as previously described [19]. Non-transfected cells were used for setting up the photomultiplier tube voltages. GFP expression was measured in the channel FL1 compensated from FL2, and DsRed-Monomer expression at the channel FL2 compensated from FL1. Channel compensations were calibrated with the cells transfected separately with each plasmid and were the same in all experiments.

For transfections with a single plasmid type, the fraction of GFP-positive cells above an arbitrarily chosen FL1 threshold was measured, as previously described [19]. For co-transfections with two plasmids, GFP fluorescence was only measured in cells that expressed high levels of DsRed-Monomer protein and therefore exhibited red fluorescence (FL2) above a threshold level of 100. This approach allowed correction for the transfection efficiencies by exclusion of non-transfected cells from analyses. Average GFP expression per transfected cell was now quantified as mean FL1 fluorescence. The results obtained were not influenced by the threshold choice as verified with the FL2 threshold levels of 30, 100 and 1000 (data not shown).

2.6. Reverse transcription and real-time PCR

Total cellular RNA was isolated with the help of TRIZOL® Reagent (Invitrogen, Karlsruhe, Germany) and treated with DNase I (Fermentas, St. Leon-Rot, Germany). DNaseI was removed by phenol extraction, RNA precipitated with isopropanol, and its integrity was confirmed by denaturing agarose gel electrophoresis. cDNA strands suitable for PCR amplification were produced by Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Efficiency of reverse transcription was confirmed with a control 1.1 kb mRNA. Oligo(dT)$_{18}$ primer and the control RNA were supplied by the kit manufacturer. cDNA samples were diluted fivefold and analysed by real-time quantitative PCR. In parallel, the samples that were not subjected to reverse transcription ("no-RT" control) were analysed in order to detect potential contamination with plasmid DNA. Real-time PCR was performed using LightCycler® 1.5 system and a LightCycler® FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). Primers specific for EGFP-