Repair of oxidative DNA damage is delayed in the Ser326Cys polymorphic variant of the base excision repair protein OGG1

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Gene–environment interactions influence an individual’s risk of disease development. A common human 8-oxoguanine DNA glycosylase 1 (OGG1) variant, Cys326-hOGG1, has been associated with increased cancer risk. Evidence suggests that this is due to reduced repair ability, particularly under oxidising conditions but the underlying mechanism is poorly understood. Oxidising conditions may arise due to internal cellular processes, such as inflammation or external chemical or radiation exposure. To investigate wild-type and variant OGG1 regulation and activity under oxidising conditions, we generated mOgg1−/− null mouse embryonic fibroblasts cells stably expressing Ser326- and Cys326-hOGG1 and measured activity, gene expression, protein expression and localisation following treatment with the glutathione-depleting compound L-buthionine-S-sulfoximine (BSO). Assessment of OGG1 activity using a 7,8-dihydro-8-oxodeoxyguanine (8-oxo dG) containing molecular beacon demonstrated that the activity of both Ser326- and Cys326-hOGG1 was increased following oxidative treatment but with different kinetics. Peak activity of Ser326-hOGG1 occurred 12 h prior to that of Cys326-hOGG1. In both variants, the increased activity was not associated with any gene expression or protein increase or change in protein localisation. These findings suggest that up-regulation of OGG1 activity in response to BSO-induced oxidative stress is via post-transcriptional regulation and provide further evidence for impaired Cys326-hOGG1 repair ability under conditions of oxidative stress. This may have important implications for increased mutation frequency resulting from increased oxidative stress in individuals homozygous for the Cys326 hOGG1 allele.

Introduction

Reactive oxygen species (ROS) are essential for cellular processes, including gene regulation, cell-mediated immunity, cell differentiation, post-translational processing of proteins and cellular signalling (1,2). ROS are generated via endogenous sources including the electron transport chain, oxidase enzymes and phagocytes as well as through environmental exposure to exogenous sources including ultraviolet (UV) light, ionising radiation, metals and polycyclic aromatic hydrocarbons. ROS can induce a range of mutagenic DNA lesions, including abasic sites, DNA strand breaks and base oxidations (3). Due to its low oxidation potential, guanine is the most easily oxidised base (4) and the two most abundant products formed are 2,6-diamino-4-hydroxy-5-formamidopyrimidine (fapyG) and 7,8-dihydro-8-oxodeoxyguanine (8-oxo dG), with subsequent oxidation reactions resulting in the formation of spiroiminodihydantoin and guanidinohydantoin (5). In normal tissues, it is estimated that the steady-state rate of formation of 8-oxo dG lesions is ~105 per cell per day and this can be as high as 105 lesions per cell per day in cancer tissues (6). 8-Oxo dG is mutagenic because it can mispair with adenine during DNA replication and lead to G:C→T:A transversion mutations (7,8). As detailed in a recent review (9), accumulation of oxidative damage has been shown to contribute to the process of normal cellular ageing (10) and various degenerative diseases, including cancer (11), Alzheimer’s disease (12), Parkinson’s disease (13) and cardiovascular disease (14).

Mammalian repair of 8-oxo dG occurs via the short patch base excision repair (BER) pathway initiated by 8-oxoguanine DNA glycosylase 1 (OGG1), a bifunctional glycosylase/AP lyase which recognises oxoG:C pairs and catalyses both the removal of 8-oxo dG and the cleavage of the DNA backbone (15). Although capable of bifunctional activity, recent evidence suggests that the AP lyase activity of hOGG1 is not essential and that the enzyme may operate as a monofunctional glycosylase in vivo (16). Subsequent activities by apurinic/apyrimidinic endonuclease 1 (APE 1), β-polymerase and DNA ligase I result in repair completion (17).

The human OGG1 gene (hOGG1) undergoes alternative splicing to generate two major isoforms: the nuclear α-hOGG1 (hOGG1-1a) and mitochondrial β-hOGG1 (hOGG1-2a) (18–20). The hOGG1 gene has been mapped to chromosome 3p26.2 (21), a region frequently subject to monoaallelic deletion and loss of heterozygosity in a number of cancers (22,23). Importantly, reduction in activity has been associated with increased risk of cancer (24,25). The mOgg1−/− null (KO) mouse is viable, but compared with wild-type has greater levels of 8-oxo dG, shows increased G:C→T:A transversion mutations in genomic DNA in non-proliferative tissues and is predisposed to lung adenocarcinoma and adenoma (26–28). There has therefore been interest in identifying mutations in the hOGG1 gene and investigating their effects on levels and activity of the protein (29).

Several polymorphisms of the hOGG1 gene have been identified (30). A single-nucleotide polymorphism at Codon 326 (S326C), present at an allele frequency of 0.33–0.45 in Asian populations and 0.22–0.27 in Caucasian populations, occurs due to a C→G substitution at position 1245 in Exon 7 and results in the exchange of a cysteine for a serine in Codon 326. Epidemiological evidence for an association between the S326C allele and cancer susceptibility is conflicting. Under normoxic conditions, individuals homozygous for the S326C
(hOGG1) allele have been shown to have an increased risk of cancers including oropharangeal, nasopharangeal, oesophageal and lung (31–37) but to have no increased risk of breast, biliary tract or colon cancers (38–42). Under normoxic conditions, reduced repair ability of S326C OGG1 has been observed by some (43,29) but not others (44–51) although catalytic efficiency ($K_{cat}/K_m$) for excision of 8-oxo dG was found to be 1.6-fold lower for purified Cys326-α-hOGG1 protein (45).

A recent analysis of epidemiology data available for low-penetrance variants in DNA repair genes and cancer susceptibility found inconclusive evidence for an association between S326C and cancer risk (52). However, as well as inherent problems such as study design differences, bias and chance, heterogeneity in epidemiological data could be due to gene–gene interactions and gene–environment interactions which are difficult to control for. Interestingly, in studies where factors including alcohol, meat consumption and smoking status were controlled for, an association between the S326C allele and increased risk of cancers including colon and stomach was observed (41,53). Furthermore, the risk of progression of *Helicobacter pylori*-induced gastric cancer, associated with oxygen-free radical formation, was increased in Cys 326 homozygotes and this risk was significantly elevated in smokers (54). This evidence suggests that any repair defect of the Cys326-hOGG1 protein becomes more apparent in oxidising conditions. In support of this, in oxidising conditions, decreased repair ability of Cys326-hOGG1 compared with wild type has been observed in cultured cells (43,55,56), using blood samples (57,58) and *in vivo* (59).

Previous work in our laboratory investigating the effect of potassium bromate on endogenous mOGG1 activity revealed that increased activity observed following exposure to potassium bromate was not a result of increased gene expression (60). Post-transcriptional OGG1 activity regulation following pro-oxidant treatment justifies the use of transgenic cell lines expressing ectopically controlled human OGG1 for investigating oxidative stress-mediated effects on OGG1 activity. A mOgg1−/− null (KO) cell line has been developed (26) and by transient hOGG1 transfection has been successfully used to investigate differences in DNA repair in oxidising conditions (56). In order to further evaluate the effect of oxidative stress on wild-type and variant hOGG1, we generated KO cells stably expressing Ser326- and Cys326-hOGG1. Oxidative stress conditions were induced using L-buthionine-S-sulfoximine (BSO), which depletes glutathione/ROS. Following aspiration of DMEM, 5 ml dimethyl sulfoxide (DMSO) was added, mixed and the absorbance was determined at 570 nm by UV spectroscopy (UVIKON Spectrophotometer 922, Kontron Instruments, UK) against a DMSO blank.

Determination of levels of ROS

Levels of total reduced glutathione were measured using a fluorescence-based technique as previously described (56,62).

**Materials and methods**

All reagents were obtained from Sigma–Aldrich (UK) unless otherwise stated.

**Cell culture**

Wild-type (WT) and KO mouse embryonic fibroblasts (MEFs) were a generous gift from T. Lindahl (Cancer Research UK, Clare Hall Laboratories, South Mimms, Hertfordshire, UK). Cells were cultured at 37°C in a humidified chamber (5% CO2, 95% air; MCO-15AC, Sanyo, Japan) in 75 cm2 cell culture flasks (Greiner Bio-one, UK) containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with foetal bovine serum (10% v/v), l-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (PAA, UK). Cells were seeded at ~1 x 105 cells per flask and passaged using a standard trypsin–ethylenediaminetetraacetic acid (0.25%/0.02%) protocol when fully confluent.

**Generation of stable cell lines**

Plasmids (pcDNA3®) containing Ser326- and Cys326-hOGG1 complementary DNA (cDNA) were a generous gift from J. Yokota (National Cancer Centre, Tokyo, Japan). These vectors were sub-cloned into a pIREShyg3 mammalian expression vector (B.D. Clontech, USA), which was used to transfect KO MEFs. Selection of Ser326- and Cys326-hOGG1 clones using hygromycin B (200 μg/ml, 120 h) was followed by confirmation of sequence identity (Functional genomics and proteomics facility, University of Birmingham, UK), hOGG1 gene expression, genomic DNA incorporation, protein expression and protein activity.

**Transfection of MEFs with molecular beacons**

Transfection with an 8-oxo dG-containing stem–loop oligonucleotide of sequence 5′-FITC-GCACAATTACGGCTGAGCGATGCAGCCCTT-CAGTGCC-DAB-3′ (where 8 is 8-oxo dG and 4-dimethylaminophenylazobenzene acid (DAB) is 4,4′-dimethylaminophenylazobenzene acid) or positive control oligonucleotide (without 8 and DAB) (Alta Biosciences, University of Birmingham, UK) was performed as described previously (60). Briefly, the beacon was diluted to 10 pmol/μl in sterile water, heated (3 min at 95°C), vortexed and left to anneal by cooling slowly (3 h) to room temperature in the dark. MEFs were transfected with beacon (140 pmol) and 2 μl transfection reagent (TuroBect, Merck, UK) according to manufacturer’s instructions. Cells were then incubated in a humidified chamber (37°C, 5% CO2) prior to analysis by flow cytometry or confocal microscopy.

**Flow cytometry analysis of fluorescein isothiocyanate-derived fluorescence in MEFs**

Samples were analysed as described previously and were not pre-selected prior to fluorescence analysis (60). CellQuestPro™ software was used to generate histograms of data, with an M-gate set at 103 on the FL1 [fluorescein isothiocyanate (FITC)] channel to define positive events. Data for populations were corrected for background fluorescence by subtracting positive events from mock-transfected populations. In order to correct for transfection efficiency, data are expressed as a percentage of the appropriate positive control–beacon positive events. Activity was defined by the number of positive fluorescent events from the 10 000 cells counted (for further explanation see supplementary Figure S1, available at Mutagenesis Online).

**Confocal microscopy**

KO MEFs were transfected with EGFPHOGG1 plasmids using TurboFect transfection reagent as per manufacturer’s instructions (Merck, UK). When indicated, cells were incubated with cytoskeletal (CSK) buffer to remove soluble proteins (63) (100 mM NaCl, 300 mM sucrose, 10 mM 1,4-piperazinediethanesulfonic acid, pH 6.8, 3 mM MgCl2, 0.5% Triton X-100 and 10 μl/mammalian protease cocktail inhibitor) for 5 min on ice prior to fixation with 4% paraformaldehyde, pH 7.4, for 20 min at room temperature. Image acquisition was performed with a Leica TCS SP confocal microscope (Leica Microsystems), using a ×63 oil immersion objective NA 1.32, Fluorochromes were excited using an argon laser at 488 nm for FITC and EGFP and 405 nm for Hoechst. Images were collected sequentially and then combined using Adobe Photoshop CS5 (Adobe Systems Inc.).

**Oxidative treatment**

Confluent cells were washed with PBS and then fresh DMEM was added containing BSO (1, 10, 100 and 1000 μM). Cells were incubated at 37°C for 24 h prior to a wash with PBS and replacement with fresh DMEM.

**Cytotoxicity assessment by MTT assay**

Cell viability was assessed by incubation with 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) at 37°C for 2 h (64). Following aspiration of DMEM, 5 ml dimethyl sulfoxide (DMSO) was added, mixed and the absorbance was determined at 570 nm by UV spectroscopy (UVIKON Spectrophotometer 922, Kontron Instruments, UK) against a DMSO blank.

**Determination of total reduced glutathione**

Levels of total reduced glutathione were measured using a fluorescence-based technique as previously described (56,62).

**Determination of levels of ROS**

ROS were measured as previously described (65) using a BD FACs Calibur utilising Cell Quest Pro software (BD), 2′,7′-dichlorodihydrofluorescein-diacetate (DCHF-DA) and the absorbance was determined at 570 nm by UV spectroscopy (UVIKON Spectrophotometer 922, Kontron Instruments, UK) against a DMSO blank.
Real-time polymerase chain reaction

Total cellular RNA was isolated from MEFs as previously described (68) using guanidinium thiocyanate detergent followed by phenol–chloroform RNA extraction and alcohol RNA precipitation (EZ-RNA kit, Geneflow). DNA contamination was removed using a DNA-free treatment kit as per manufacturer’s instructions (Ambion). Total RNA (1 μg) was used for first-strand cDNA synthesis using a SuperScript II Reverse Transcriptase kit as per manufacturer’s instructions (Invitrogen). OGG1 cDNA was quantified by spectrophotometry using a Nanodrop ND1000 and then used in polymerase chain reaction (PCR) assays using a Sensimix dT SYBR Green kit (Quintace, Finchley, UK) and OGG1 primers (Forward: 5′-AGGGTGCGAGGCTCATCTCAG-3′; Reverse: 5′-AGGGTGCCAGCTGTAGTCAC-3′). Per well, cDNA (200 ng) was combined with 13 μl Sensimix, 8 μl dH2O, 0.5 μl SYBR Green and 0.2 pmol/μl primers and made up to a final volume of 25 μl. Real-time PCR was performed with all samples (n = 3) in duplicate, using an Ambiprim 7000 sequence detection system with products amplified and detected, following a dissociation protocol, using a programme of 95°C for 30 s and 61.1°C for 30 s.

PCR products were confirmed by sequencing (Functional Genomics and Proteomics Facility, University of Birmingham, UK). The melt curves for all samples were analysed and Ct values were recorded for each gene in the linear phase of amplification. Differences in sample Ct values (n = 3) were assessed using a one-way analysis of variance (ANOVA) with a Tukey post-hoc test (SPSS v16). PCR efficiencies were assessed using the LinRegPCR software (69).

Preparation of protein extracts

Whole cell extracts were prepared by incubating cell pellets (~5 × 10⁶ cells) with radioimmunoprecipitation buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40 (v/v), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) (w/v), 10 μl/ml mammalian protease cocktail inhibitor) on ice for 20 min with regular vortexing. Cell debris was pelleted by centrifugation at × 14 000 for 15 min at 4°C. Protein content was determined by the method of Bradford (70) using bovine serum albumin as a standard.

Western blotting

Protein extracts (70 μg) were boiled in Laemmli sample buffer for 5 min and resolved on a 12.5% SDS–polyacrylamide gel, transferred to a polyvinylidene fluoride membrane (Amersham Biosciences) and blocked in blocking buffer (5% low-fat powdered milk (Marvel) in 1× TBS–0.05% Tween 20) for 1 h at 4°C. Membranes were incubated with a rabbit polyclonal anti-GFP primary antibody (1:2000 dilution; Abcam, Cambridge, UK) in blocking buffer for 1 h at room temperature, washed in TBS–0.05% Tween 20 solution (3 × 10 min) and then incubated with a horseradish peroxidase-conjugated polyclonal goat anti-rabbit secondary antibody (1:5000 dilution; Dakocytomation, UK) in blocking buffer for 1 h. Following washing, protein bands were detected by incubation with SuperSignal West Pico chemiluminescent detection reagent (Thermo Fisher Scientific, UK), prepared as per manufacturer’s instructions and visualised via exposure to ECL hyperfilm (Amersham Biosciences). The intensity of each band was quantified by densitometry (ImageJ) and normalised to a beta actin loading control.

Statistical analysis of data

All statistical analysis was performed using SPSS version 16. Data that were normally distributed (as assessed by Shapiro–Wilk test) and displayed homogeneity of variance (as assessed by Levene’s test) were analysed by one-way ANOVA followed by a two-tailed or paired Student’s-t test. Data that were not normally distributed or did not display homogeneity of variance were analysed by Kruskal–Wallis test or Mann–Whitney U-test. All data are displayed ±SEM from at least three independent experiments.

Results

Generation of Ser326- and Cys326-hOGG1 MEFs

To investigate the effect of oxidative stress on hOGG1 activity, cell lines expressing either Ser326- or Cys326-hOGG1 protein were established by the introduction of a cDNA expression vector for each form of OGG1 into KO MEFs. OGG1 gene expression, protein expression and activity, absent in KO MEFs, were detected in both Ser326- and Cys326-hOGG1 MEFs (data not shown). There was no statistically significant difference in Ser326- and Cys326-hOGG1 gene or protein expression (data not shown) and no statistically significant difference in basal Ser326- and Cys326-hOGG1 activity, F = 0.34 (supplementary Figure S2, available at Mutagenesis Online).

Treatment of MEFs with non-cytotoxic concentrations (0–1000 μM) of BSO depleted reduced glutathione and induced ROS

BSO is a specific and potent inhibitor of reduced glutathione (GSH) synthesis (61) and has been previously used to deplete GSH from the nucleus and mitochondria of mOGG1+/− and KO MEFs at concentrations ≥100 μM (62). Cell viability following BSO treatment (0–1000 μM) was assessed by MTT assay and was >95% (supplementary Figure S3, available at Mutagenesis Online). The current study therefore used these non-cytotoxic concentrations (0–1000 μM) to deplete GSH within KO and Ser326- and Cys326-hOGG1 MEFs (Figure 1A). As expected, GSH depletion resulted in a subsequent increase in ROS, as assessed by the oxidation of H2DCF, in KO and Ser326- and Cys326-hOGG1 MEFs (Figure 1B). There was no statistically significant difference between cell lines in either GSH depletion or ROS production following BSO treatment. Following removal of BSO, GSH levels remained depleted (50% of controls) 24 h post-treatment, and ROS levels remained elevated (136% of controls) 24 h post-treatment.

Oxidative stress induced by BSO treatment resulted in an increase in OGG1 activity, which occurred earlier in Ser326-hOGG1 MEFs

The Cys326-hOGG1 variant has been previously shown to have reduced repair activity compared with wild type under conditions of oxidative stress (43,56,58,59). The current study used an 8-oxo dG-containing molecular beacon to observe and quantify OGG1 activity in live cells in real time. The beacon assay is specific for OGG1 activity, as demonstrated previously with negligible fluorescence observed following transfection of KO MEFs (60). To investigate the effect of GSH depletion and ROS induction by BSO treatment on the activity of Ser326- and Cys326-hOGG1, MEFs were treated with BSO (1000 μM, 24 h), washed and then transfected with 8-oxo dG-containing molecular beacon. Quantification by flow cytometry of the fluorescence resulting from OGG1-mediated beacon cleavage every 4 h post-treatment revealed that the activities of both Ser326- and Cys326-hOGG1 were increased by BSO treatment (representative histograms are shown in supplementary Figure S4, available at Mutagenesis Online). The peak activity of Ser326-hOGG1 occurred 12 h prior to that of Cys326-hOGG1 (Figure 2A). A concentration–response relationship between activity and BSO concentration was observed for both Ser326- and Cys326-hOGG1 (Figures 2B and 2C). The activity increase was confirmed in live cells using confocal microscopy 12 h post-treatment (supplementary Figure S5, available at Mutagenesis Online) and 24 h post-treatment (supplementary Figure S6, available at Mutagenesis Online) and revealed that although background cytoplasmic beacon fluorescence was observed, the location of the cleaved beacon was predominantly nuclear.

To confirm beacon fluorescence was specific to OGG1 activity, KO MEFs were transfected with 8-oxo dG-containing molecular beacon. Negligible background fluorescence and no
Fig. 1. BSO depleted GSH and induced ROS in MEFs. (A) Concentration-dependent decrease in reduced glutathione following 24-h BSO treatment. Data are displayed as percentage of untreated control (nm GSH/mg protein, KO MEF: 18.54 ± 1.32, Ser326-hOGG1 MEF: 24.77 ± 1.77, Cys326-hOGG1 MEF: 19.88 ± 2.30, n = 3) (*, ** and ***: significantly different from control *P < 0.05, **P < 0.01 and ***P < 0.001 as determined by two-tailed Student’s t-test). (B) Concentration-dependent increase in ROS, as assessed by DCF fluorescence, following 24-h BSO treatment (n = 4) (**significantly different from control *P < 0.05 as determined by Mann–Whitney U-test).

Increase in OGG1 activity was not due to increased gene expression or protein levels

Increased mouse OGG1 activity in MEFs following exposure to potassium bromate was not associated with any change in gene expression (60). In the current study, the expression of ser326- and cys326-hOGG1 is under the control of the cytomegalovirus (CMV) promoter. To ensure that BSO had no effect on the CMV promoter, real-time PCR was used to measure the gene expression levels of both ser326- and cys326-hOGG1 at 6-h intervals during the 24-h BSO treatment period (1000 μM). As expected, there was no change in gene expression indicating that the observed increases in OGG1 activity are not a result of transcriptional activation (Figure 3).

To investigate the potential effects related to protein stabilisation, KO MEFs were transiently transfected with plasmids expressing EGFP-Ser326-hOGG1 or EGFP-Cys326-hOGG1. Following BSO treatment (1000 μM, 24 h), protein levels were measured by western blot 0, 12 and 24 h post-treatment with beta actin used as a loading control. Densitometry values, shown as absorbance units (AU), are expressed as a ratio of treated AU to time-matched control AU. Although both Ser326- and Cys326-hOGG1 protein amounts increased slightly 12 and 24 h post-treatment, no statistically significant change in protein levels was observed between BSO treated and control samples.

Table 1. Negligible beacon cutting activity in KO MEFs following BSO treatment

<table>
<thead>
<tr>
<th>BSO (μM)</th>
<th>KO MEFs ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.52 ± 0.13</td>
</tr>
<tr>
<td>100</td>
<td>0.68 ± 0.29</td>
</tr>
</tbody>
</table>

The fluorescence (number of positive events) did not significantly increase following BSO treatment and was negligible compared with that seen in Ser326- and Cys326-hOGG1 MEFs (n = 3) (P > 0.05 as determined by two-tailed Student’s t-test).
DNA damage repair by Ser326Cys OGG1 is delayed

Table II. No change in positive control beacon fluorescence in Ser326- and Cys326-hOGG1 MEFs following BSO treatment

<table>
<thead>
<tr>
<th>BSO (µM)</th>
<th>Ser326-hOGG1 MEFs (12 h post-treatment)</th>
<th>Cys326-hOGG1 MEFs (12 h post-treatment)</th>
<th>Ser326-hOGG1 MEFs (24 h post-treatment)</th>
<th>Cys326-hOGG1 MEFs (24 h post-treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>72.03 ± 3.19</td>
<td>74.11 ± 5.10</td>
<td>78.63 ± 8.05</td>
<td>73.63 ± 7.71</td>
</tr>
<tr>
<td>1</td>
<td>71.86 ± 4.47</td>
<td>71.44 ± 6.70</td>
<td>69.51 ± 12.14</td>
<td>71.74 ± 10.27</td>
</tr>
<tr>
<td>10</td>
<td>70.10 ± 2.53</td>
<td>69.73 ± 5.37</td>
<td>69.36 ± 17.27</td>
<td>64.45 ± 16.06</td>
</tr>
<tr>
<td>100</td>
<td>72.90 ± 5.67</td>
<td>70.58 ± 5.67</td>
<td>66.41 ± 18.81</td>
<td>64.72 ± 15.44</td>
</tr>
<tr>
<td>1000</td>
<td>69.37 ± 4.81</td>
<td>67.83 ± 8.62</td>
<td>67.98 ± 16.67</td>
<td>68.89 ± 16.09</td>
</tr>
</tbody>
</table>

Transfection efficiency, as assessed by the number of positive control beacon fluorescent events, did not vary between the two cell types and was not affected by BSO treatment (n = 3) (P > 0.05 as determined by one-way ANOVA).

Fig. 3. hOGG1 gene expression did not change following BSO treatment (1000 µM). RNA was isolated from control and BSO-treated Ser326- and Cys326-hOGG1 MEFs, cDNA (200 ng per well) was used to quantify hOGG1 gene expression (n = 3). No statistically significant difference in Ct values was observed (P > 0.05 as determined by one-way ANOVA).

Fig. 4. hOGG1 protein levels did not change significantly following BSO treatment (1000 µM). (A) Western blot analysis of BSO-treated EGFP-Ser326-hOGG1 and EGFP-Cys326-1 hOGG1-expressing MEFs. (B) Quantification of signals by densitometry from three independent experiments using ImageJ, with values normalised to the beta actin loading control. Each value represents a treated sample as a ratio of time-matched control, ±SEM. No significant difference in protein levels was observed (P > 0.05 as determined by one-way ANOVA).

Ser326- and Cys326-hOGG1 proteins localised to the nucleus (supplementary Figure S9, available at Mutagenesis Online). No differences in localisation were observed following BSO treatment. Wild-type hOGG1-GFP protein has previously been shown to remain soluble in untreated cells but to be retained in an insoluble nuclear fraction following treatment with the pro-oxidant potassium bromate (63). To investigate whether there was a difference between retention of Ser326- and Cys326-hOGG1, in this insoluble nuclear fraction following BSO treatment, KO MEFs were transiently transfected with plasmids expressing EGFP-Ser326-hOGG1 or EGFP-Cys326-hOGG1. Following BSO treatment (1000 µM, 24 h), cells were incubated with CSK buffer to remove soluble proteins (63), washed and then fixed with paraformaldehyde. Any remaining insoluble EGFP-hOGG1 was observed by confocal microscopy. In agreement with Amouroux et al. (63), we observed retention of Ser326-hOGG1, compared with untreated cells, within the detergent-resistant fraction following BSO treatment (supplementary Figure S10, available at Mutagenesis Online). Interestingly, Ser326-hOGG1 was retained within the insoluble nuclear fraction to a greater extent than Cys326-hOGG1. There was no difference between EGFP-hOGG1 levels prior to the CSK wash (supplementary Figure S11, available at Mutagenesis Online).

Discussion

Inflammation, exposure to cigarette smoke, ionising radiation, environmental pollution, toxic food ingredients and sunlight can all result in increased cellular ROS and increased 8-oxo dG formation (72). Rapid and efficient repair of 8-oxo dG from...
genomic DNA is required to prevent errors during DNA replication and elevated frequency of mutation and cancer. An increased risk of cancer development has been observed in individuals homozygous for the S326C hOGG1 allele, particularly when external factors including smoking and diet are controlled for (32,73). It is accepted that in addition to the individual contributions of genetic differences and environmental factors, interactions between the two are important in disease development (74) and evidence supports an interaction between the Cys326 hOGG1 allele and environmental exposure to ROS resulting in increased risk of cancer (53,75). In support of this, it is becoming increasingly clear that reduced repair ability of Cys326-hOGG1 becomes more apparent under oxidising conditions (43,55–59).

In order to quantitatively assess and visualise OGG1 repair activity in real time, we have optimised a molecular beacon assay based on work by Maksimenko et al. (76) which can be used in live cells to measure OGG1 activity (60,77). The current study used this technique to compare Ser326- and Cys326-hOGG1 activity after treatment with the glutathione synthesis inhibitor BSO. In this study, Ser326- and Cys326-hOGG1-expressing KO MEF cells were developed, with comparable beacon cutting activity in the absence of oxidative stress (supplementary Figure S2, available at Mutagenesis Online), treated with BSO to deplete GSH (Figure 1A) and induce ROS (Figure 1B) and used to show that the response of Ser326-hOGG1 to oxidative stress conditions was faster than that of Cys326-hOGG1 (Figure 2).

The response of OGG1 to oxidative stress has been previously investigated with both induction and reduction of activity observed (60,78–86). Reduction of OGG1 activity under conditions of oxidative stress would result in reduced DNA repair capacity and sensitivity to chemically induced oxidative DNA damage. We show here that the activity of both Ser326-hOGG1 and Cys326-hOGG1 increased following BSO-induced ROS accumulation (Figure 2) but that this increase was not related to any gene expression or protein increase (Figures 3 and 4).

The expression of hOGG1 does not vary during the cell cycle and this, combined with the lack of TATA or CAAT boxes within the promoter, indicates that it is a constitutively expressed house-keeping gene (87). The OGG1 promoter does however contain SP1 transcription factor-binding sites and a Nrf2 antioxidant response element and expression of OGG1 can be modulated by a range of cellular stresses (79–81,88,89). In the current study, the expression of ser326- and cys326-hOGG1 was under the control of the CMV promoter and therefore not subject to regulation at the transcriptional level. These data support other studies which show that oxidative stress has the ability to modulate OGG1 activity post-translationally (60,82,83,86). We did not find a consistent statistically significant change in Ser326- and Cys326-hOGG1 protein levels following BSO treatment (Figure 4 and Supplementary Figure S9, available at Mutagenesis Online) therefore, although we cannot fully exclude the possibility due to variability observed in our repeat experiments, our data show that protein stabilisation does not completely explain increased hOGG1 activity.

As Ser326- and Cys326-hOGG1 protein levels remained largely unchanged following BSO treatment, it is therefore possible that post-translational modifications are responsible for the activity modulation observed in our study as it has been shown that OGG1 activity can be stimulated by phosphorylation (90) and acetylation (82). It is possible that the slight band shift observed in Ser326-hOGG1 T12 samples and Cys326-hOGG1 T24 samples (Figure 4) may reflect post-translational modification. Much further work remains to be done to investigate the phosphorylation and acetylation status of the protein following oxidative stress. The process of BER is highly coordinated and involves multiple interactions between the proteins of the pathway. The activity of OGG1 has previously been shown to be enhanced by apurinic/apyrimidinic endonuclease 1 (APE1) (91,92), NEL-like glycosylase 1 (NEIL1) (93), X-ray cross complementing protein 1 (XRCC1) (94) and RAD52 (95). It is therefore also possible that oxidative stress induced by BSO treatment could affect the interaction with one of these protein partners.

The human OGG1 gene undergoes alternative splicing generating two major isoforms: α-hOGG1 (hOGG1-1a) and β-hOGG1 (hOGG1-2a). The first 316 amino acids are identical and contain a mitochondrial targeting sequence; however, the carboxy-terminal ends differ extensively, with that of α-hOGG1 containing a C-terminal nuclear localisation sequence absent from β-hOGG1 (18) which targets it to the nucleus (19) and suppresses the mitochondrial targeting sequence which targets β-hOGG1 to the mitochondria (20). Little is known about the function of the β-form; as surprisingly, it has been shown to lack repair activity (96) but to have an effect on the mechanism of cell death in MEFs (97). Although mainly located in the nucleus, it is thought that the alpha form is responsible for both nuclear and mitochondrial 8-oxo dG repair. The current study used MEFs expressing the α-hOGG1 isoform and analysis by confocal microscopy revealed that, as expected, in both Ser326- and Cys326-hOGG1 MEFs the cleaved beacon was mainly located in the nucleus (supplementary Figures S5 and S6, available at Mutagenesis Online). Furthermore, fluorescence of nuclear localised beacon increased in intensity in a concentration-dependent manner following BSO treatment. Interestingly, this is in contrast to previous observations, which showed mitochondrial beacon localisation following treatment of MEFs expressing endogenous mOGG1 with potassium bromate (60). Mouse OGG1 exists as only a single isoform responsible for both nuclear and mitochondrial BER. The staining pattern observed using the positive control beacon (supplementary Figures S7 and S8, available at Mutagenesis Online) shows that in the current study, the beacon is present in the nucleus, cytoplasm and mitochondria. Based on these observations, we propose that the localisation difference may either be a species-specific response or a result of hOGG1 overexpression. Alternatively, it is possible that in these cells, in contrast to potassium bromate treatment, it is genomic rather than mitochondrial DNA that is preferentially oxidatively damaged following BSO treatment, resulting in an accumulation of cleaved beacon in the nucleus, the site of greatest damage.

An increase in OGG1 activity following increased intracellular ROS is easily rationalised as a response to the requirement of the cell to repair oxidatively damaged DNA. Any inability to rapidly repair 8-oxo dG lesions results in the accumulation of G:C→T:A transversion mutations; among the most predominant somatic mutations in lung, breast, ovarian, gastric and colorectal cancers (98) and frequent in the mutational spectrum of the tumour suppressor gene p53 (99). Our data show that this increased activity response is delayed in the Cys326-hOGG1 variant (Figure 2). To ensure that this was not a clone-specific effect, we repeated the activity
assessments using two independent MEF cell lines expressing Ser326- and Cys326-hOGG1 and observed the same trend (data not shown). Cys326-hOGG1 has previously been shown to have decreased 8-oxo dG-binding affinity and reduced 8-oxo dG excision catalytic efficiency (45,55). Interestingly, although Cys326-hOGG1 was less efficient than wild-type hOGG1 removing 8-oxo dG:C from oligodeoxynucleotide substrates, the effect was far more pronounced when removing 8-oxo dG from high-molecular weight DNA (100).

Our data are in agreement with functional studies observing reduced rate of repair of Cys326-hOGG1 in oxidising conditions and thus support epidemiological evidence suggesting increased risk of cancer in individuals homozygous for the Cys326-hOGG1 allele, particularly under conditions of oxidative stress. Although the genetic risks of cancer development conferred by this single gene polymorphism may be modest (52), our data further support Bravard et al. (43) who noted that a difference in redox environment between cases and controls may explain some variability in epidemiological studies. In addition, it is important to consider that DNA repair does not occur by a single enzyme in isolation and there is cross-talk between repair pathways. As polymorphisms exist in a variety of DNA repair enzymes, an individual with a combination of mutant repair proteins could be at a greater risk of disease development.

The mechanism underlying the Cys326-hOGG1 repair difference is unknown and as no crystal structure exists that includes amino acids beyond position 325 no information can be obtained from structural studies. Two major hypotheses have been proposed to explain repair differences: the introduction of a redox-sensitive cysteine residue or the loss of a serine residue whose phosphorylation may be important for OGG1 localisation and activity. An increasing body of evidence supports the theory that the cys326 residue, present in a highly positively charged sequence environment (ADLRQ[ser326cys]RHAQ), is redox sensitive and susceptible to the formation of a reactive thiolate anion (101) which can be oxidatively modified and form disulfide bonds (43). The cys326 residue is present in a disordered and highly mobile region (15), suggesting that this residue may be located in an accessible part of the molecule, rendering it a likely target for redox modification. Hill and Evans (55) showed that purified Cys326-hOGG1 exists as a dimer, which may explain a reduction in activity; however, work in our laboratory using bimolecular fluorescence complementation assays has failed to demonstrate hOGG1 dimer formation in unstressed or stressed live cells (data not shown).

Our data show that, as expected, the localisation of both EGFP-Ser326- and EGFP-Cys326-hOGG1 in MEFs was predominantly nuclear (supplementary Figure S9, available at Mutagenesis Online). The localisation of the proteins did not change either 12 or 24 h post-treatment, when the activity difference was detected, suggesting that BS0 treatment does not affect the localisation of Ser326- or Cys326-hOGG1 and that the activity induction was not as a result of any change in hOGG1 cellular localisation. Previous studies have shown that the subnuclear localisation of OGG1 varies throughout the cell cycle (71,102). During interphase, hOGG1 is associated with the soluble chromatin and nuclear matrix, whereas during mitosis, it associates with condensed chromosomes and relocalises to the nucleoli during S-phase. All these relocalisation processes have been shown to be mediated through the phosphorylation of serine326, with the subcellular localisation of Cys326-hOGG1 disrupted by exclusion from the nucleoli during S-phase (71). There is increasing interest in the subnuclear distribution of BER proteins following oxidative stress but our data do not support any difference in hOGG1 localisation to the nucleus or nucleoli between the two proteins or following BS0 treatment.

Interestingly, hOGG1 has been shown to be recruited to nuclear speckles via a ROS-mediated mechanism (103) and to rapidly accumulate at sites of laser irradiation-induced DNA damage (104). A recent report also demonstrated that hOGG1 is recruited to regions of open chromatin following exposure to high concentrations of potassium bromate. This study showed that in unstressed cells, hOGG1 exists in a soluble cellular fraction containing the cytoplasm and nucleoplasm. Following treatment with high concentrations of potassium bromate, hOGG1, along with APE1 and XRCC1, is recruited to and retained within an insoluble cellular fraction containing chromatin- and matrix-associated proteins (63). This recruitment was not cell cycle dependent. Our data support the finding that OGG1 was concentrated on chromatin regions and suggests that following oxidative stress wild-type (Ser326) OGG1 is more stably associated with chromatin than Cys326 OGG1 (supplementary Figure S10, available at Mutagenesis Online). Although further confirmation is required, we hypothesise that this difference plays a role in the delayed repair observed.

In conclusion, although the mechanism of delayed repair and reduced nuclear retention of Cys326 is currently unknown, the data presented here provide further evidence for impaired Cys326-hOGG1 repair ability under conditions of cellular oxidative stress which may have important implications for individual susceptibility to increased cellular oxidative stress.

Supplementary data
Supplementary Figures S1–S11 are available at Mutagenesis Online.

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