Glutathione depletion by buthionine sulfoximine induces DNA deletions in mice

Ramune Reliene and Robert H. Schiestl

Department of Pathology, Department of Environmental Health and
Department of Radiation Oncology, Geffen School of Medicine and
School of Public Health, UCLA, Los Angeles, CA 90024, USA

1To whom correspondence should be addressed at: Department of Pathology, UCLA School of Medicine, 650 Charles E. Young Drive South, Los Angeles, CA 90024, USA. Email: rschiestl@mednet.ucla.edu

Oxidative stress and genomic rearrangements play a role in cancer development. 1-Buthionine-sulfoximine (BSO) induces oxidative stress in a cell by irreversibly inhibiting \(\gamma\)-glutamylcysteine synthetase, an essential enzyme for the synthesis of glutathione (GSH). We postulated that oxidative stress induced by GSH depletion might lead to genomic rearrangements, such as DNA deletions, and that counteracting such pro-oxidant conditions by the exogenous antioxidant N-acetyl-L-cysteine (NAC), might suppress DNA deletions. Therefore, we determined the frequency of 70 kb DNA deletions and thiol levels in mouse fetuses exposed to BSO (alone or in combination with NAC) via drinking water given to female mice during gestation. BSO treatment resulted in a significantly increased frequency of DNA deletions and decreased concentrations of GSH and cysteine. An amount of 2 mM BSO treatment resulted in a 30% higher DNA deletion frequency, 45% lower GSH and 27% lower cysteine levels, when compared with the untreated control and 20 mM BSO treatment caused a 40% higher DNA deletion frequency, 70% lower GSH and 55% lower cysteine levels. In combination BSO and NAC resulted in reduced levels of GSH consistent with the effect of BSO; however, cysteine levels increased and the frequency of DNA deletions was within the normal range. Thus, NAC protected against genome rearrangements caused by GSH depletion. This study showed that lowering the concentrations of thiol antioxidants results in DNA deletions that may play a role in carcinogenesis.

Introduction

Oxidative phosphorylation used for energy production in all heterotrophic eukaryotes is accompanied by leakage of reactive oxygen species (ROS) from mitochondria to the cytosol and \(\sim 10^6\) and \(10^5\) oxidative damage hits to DNA bases per cell in human and rat cells, respectively (1). Oxidative stress has been associated with cancer and other diseases, such as neurodegenerative diseases, cardiovascular diseases and cataracts, to name a few (1). It has been suggested that oxidative stress contributes to cancer development by modulating signal transduction pathways related to cell growth, differentiation and cell death and by increasing the gene mutation rate (1,2). For instance, a lesion formed from oxidative damage to guanine (G) residue, 8-OH deoxyguanosine (8-OHdG), frequently mismatched with an adenine (A) base (3) causing \(GC\rightarrow TA\) transversions. However, most human tumors are characterized by large genome rearrangements, such as DNA deletions, translocations and duplications (4,5). Such genome rearrangements are regarded as being involved in carcinogenesis although the mechanism of genome rearrangements in this process is not well understood. Presumably, some of these genomic events might be mediated by oxidative stress.

Low molecular weight thiol-containing molecules, such as glutathione (GSH; \(L-\gamma\)-glutamyl-L-cysteinyl-glycine) and cysteine, protect against oxidative stress. GSH is the most abundant intracellular antioxidant having an important role in the protection against ROS, metabolism of nutrients and xenobiotics, and regulation of intracellular redox status (6,7). GSH is a major source of intracellular reductant and it regenerates oxidized vitamins to their reduced form (8). There is evidence for a link between GSH and aerobic metabolism evolution in eukaryotes, which can be explained by the role of GSH in protecting against oxygen toxicity (9). The role of GSH is often studied under conditions that deplete intracellular GSH. Buthionine sulfoximine (BSO) depletes GSH and thereby induces oxidative stress by specifically and irreversibly inhibiting \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)-GCS), the rate-limiting enzyme of GSH synthesis (10). Administration of BSO leads to decreased GSH levels in virtually all tissues including developing embryos, and a marked GSH depletion is associated with tissue damage (11–16). Multiorgan failure and death occur when BSO is administered to newborn rats (17,18). Additionally, pretreatment with BSO enhances the toxicity of radiation and drugs (12). A non-toxic analog of cysteine, \(N\)-Acetyl cysteine (NAC), is readily taken up by cells and is used for GSH synthesis or it can directly scavenge ROS (19,20). Thus, NAC may be protective against BSO-induced oxidative stress. In fact, \(\gamma\)-GCS deficient cells undergo apoptosis, but can be propagated indefinitely in the presence of NAC (21). In a developing mouse, NAC suppresses formation of 8-OHdG DNA lesions and bulky DNA adducts resulting from oxidative stress occurring at birth (22) or following exposure to cigarette smoke (23).

We hypothesized that oxidative stress may play a role in carcinogenesis by possibly leading to large genome rearrangements. To test this hypothesis we determined the frequency of DNA deletions and concentrations of thiol antioxidants, GSH and cysteine, in mouse fetuses exposed to BSO (alone or in combination with NAC) via drinking water given to pregnant dams. We determined the frequency of 70 kb DNA deletions resulting from intrachromosomal homologous recombination between two 70 kb tandem repeats at the 'pink-eyed unstable'...
Materials and methods

Mice and treatment
C57BL/6J p<sup>un</sup>/p<sup>un</sup> mice were obtained from the Jackson Laboratory (Bar Harbor, ME). BSO and NAC were purchased from Sigma (St Louis, Mo). Mice were bred in the institutional specific pathogen free animal facility under standard conditions with a 12 h light/dark cycle and were fed standard diet and water ad libitum. Pregnancy was timed by checking for vaginal plugs. Noon of the day of discovery was counted as 0.5 days post partum (d.p.p.). Similarly, the time of birth of a litter was timed with the noon of discovery counted as 0.5 days post partum (d.p.p.).

NAC and BSO were purchased from Sigma. Pregnant dams were given free access to drinking water supplemented by either 2 mM BSO, 20 mM BSO, 2 mM BSO and 20 mM NAC, 20 mM NAC or unsupplemented water for 18 days from 0.5 to 18.5 d.p.c. The pH of supplemented water was as follows: 6.88, 20 mM BSO; 3.37, 2 mM BSO; 2.65, 2 mM BSO plus 20 mM NAC; and 2.58, 20 mM NAC. The pH of regular water used in our facility is ~4 (it is hydrochloric acid treated to prevent microbial infection). Intake of supplemented and unsupplemented water was similar, averaging to 5 ml not accounting for spill and yielding a daily intake of BSO 0.1 g/kg (0.45 mM/kg) or 1 g/kg (4.5 mM/kg) when administered at 20 or 20 mM, respectively. These BSO doses were chosen, because comparable BSO doses (intake of 2-6 mM/kg/day) administered to pregnant rats in drinking water throughout pregnancy lowered GSH levels but had no teratogenic effects in offspring (34). NAC daily intake was ~0.5 g/kg, similar to the NAC concentration we used in a previous study (30).

To determine the DNA deletion frequency, 20-day-old offspring (23 mice in the control group and 16–17 mice per exposure group) were sacrificed to visualize eye-spots (DNA deletions) in their RPE. For thiol determination the control group and 16–17 mice per exposure group were sacrificed to administer to pregnant rats in drinking water throughout pregnancy lowered (4.5 mM/kg) when administered at 2 or 20 mM, respectively. These BSO doses were chosen, because comparable BSO doses (intake of 2-6 mM/kg/day) administered to pregnant rats in drinking water throughout pregnancy lowered GSH levels but had no teratogenic effects in offspring (34). NAC daily intake was ~0.5 g/kg, similar to the NAC concentration we used in a previous study (30).

To determine the DNA deletion frequency, 20-day-old offspring (23 mice in the control group and 16–17 mice per exposure group) were sacrificed to visualize eye-spots (DNA deletions) in their RPE. For thiol determination, mouse fetuses from 3 to 4 dams per exposure scenario were isolated at 17.5 d.p.c. Thiol levels were determined in whole-body fetuses, but not in the isolated RPE, because it would not yield sufficient amount of tissue (50–100 mg) required for thiol determination. The RPE is a single cell layer consisting of only ~50 000 cells in total (35).

Dissection of the retinal pigment epithelium
Offspring were sacrificed at 20 days of age and their eyes were dissected. Whole mount RPE slides were prepared for microscopic analysis of eye-spots. Eyes were processed to expose the RPE layer as previously described (29,35,36). The eye was removed from its orbit and immersed in fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for 1 h and then in phosphate buffered saline (PBS) until dissection. An incision was made at the upper corneal–scleral border to allow removal of the cornea and lens. To flatten the eye-cup, six to eight incisions were made from the corneal–scleral margin towards the centrally positioned optic nerve, and the dissected eye cup was placed on a glass slide with the retina facing up. The retina was then gently removed and the residual specimen consisting of sclera, chorioid and RPE, with the RPE facing up, was mounted in 90% glycerol for microscopic analysis.

Scoring single deletion events, visualized as eye-spots
A pigmented cell or a group of adjacent pigmented cells separated from each other by no more than five unpigmented cells was considered as one eye-spot that resulted from one deletion (p<sup>un</sup> reversion) event (43).

Determination of GSH and cysteine content
Mouse fetuses were isolated at 17.5 d.p.c., immediately frozen in liquid nitrogen and homogenized under liquid nitrogen. Approximately 50 mg of tissue was suspended in 200 μl of PBS and an equal volume of freshly prepared 10% metaphosphoric acid was added (pH 1.8), mixed by vortexing and incubated for 30 min on ice followed by centrifugation for 15 min at 14000 r.p.m. The supernatant was used for thiol determination and the pellet was dissolved in 1 M NAOH by incubating at 37°C for 1 h for determination of protein concentration. Protein concentration was determined using Bio-Rad’s DC protein assay (Lowry method) according to manufacturer recommendations (Bio-Rad laboratories, Hercules, CA). Thiol concentrations were expressed in micrograms of compound per milligram of protein. The thiol levels were determined by HPLC chromatography coupled to coulometric electrochemical (EC) detection as described before (38). Brieﬂy, 20 μl of the supernatant diluted 15- and/or 30-fold with mobile phase was injected into the HPLC column. The HPLC-EC detection system consisted of an Agilent Technologies 1100 binary pump, autosampler, variable wavelength detector, controlled by Chemstation Software 7.01 (Agilent Technology, San Diego, CA), an ESA Coulochem II electrochemical detector (ESA, Bedford, MA), Supelco LC-18 column, 150 x 4.6, 5 μm (Sigma-Aldrich, St Louis, MO). The mobile phase consisted of 50 mM sodium phosphate monobasic monohydrate, 1.0 mM ion-pairing reagent octane sulfonic acid, 2% acetoniﬁre (v/v) adjusted to pH 2.7 with 85% phosphoric acid. Elution was isocratic at a flow rate of 1.0 ml/min. Levels were quantified using the standard curves of the tested compounds.

Statistical analysis
The number of eye-spots and thiol concentrations were compared using an unpaired Student t-test.

Results

The frequency of DNA deletions in BSO treated mice
The frequency of DNA deletions in mouse offspring treated with BSO during embryonic development was examined as the number of eye-spots in the RPE. There were four treatment groups and a control (untreated) group. The average number of eye-spots (mean ± SEM) was 5.36 ± 0.29 (n = 46), 7.79 ± 0.45 (n = 34) and 8.78 ± 0.61 (n = 32) in untreated controls, 2 mM BSO and 20 mM BSO treated mice, respectively (Figure 2). The 2 mM BSO treatment resulted in ~30% more eye-spots (P < 0.001), and the 20 mM treatment resulted in 40% more eye-spots (P < 0.001) compared with untreated mice. This finding demonstrated that BSO causes an elevated frequency of DNA deletions during mouse development.

The effect of NAC co-treatment was determined using the 2 mM BSO dose. Mice treated simultaneously with 2 mM BSO and 20 mM NAC had signiﬁcantly less (P = 0.016) eye-spots than those treated with 2 mM BSO, such as 6.18 ± 0.47 versus 7.79 ± 0.45 eye-spots (n = 34 in each group), indicating that NAC prevented against DNA deletions (Figure 2). The 20 mM NAC treatment group, used as a negative control for the BSO plus NAC treated mice, had similar number of eye-spots...
The effect of co-treatment was also compared with untreated mice and showed no significant difference between the groups, implying that NAC counteracted the effect of BSO. There was also no significant difference between the untreated and 20 mM NAC treated mice.

GSH concentration in BSO treated mice
BSO treatment reduced GSH concentration in mouse fetuses by 55 (P < 0.01) and 70% (P < 0.001) at 2 mM and 20 mM BSO doses, respectively, compared to untreated mice (Figure 3). Co-treatment with 2 mM BSO and 20 mM NAC depleted GSH to a similar extent (P < 0.01) as 2 mM BSO, consistent with the function of BSO to inhibit the γ-GCS enzyme indispensable for GSH synthesis (Figure 3). This finding demonstrated that NAC suppressed DNA deletions in the BSO plus NAC treatment group (shown in Figure 2) directly and not by replenishing GSH pools. In 20 mM NAC treated mice, GSH levels increased by 30% (P < 0.05) (Figure 3) and the frequency of DNA deletions was slightly lower (10%) compared to untreated mice (shown in Figure 2), although the difference was not statistically significant. Apparently, an increase in GSH level above the normal level does not reduce the spontaneous frequency of rearrangements.

GSH concentration in BSO treated mice
BSO treatment reduced GSH concentration in mouse fetuses by 55 (P < 0.01) and 70% (P < 0.001) at 2 mM and 20 mM BSO doses, respectively, compared to untreated mice (Figure 3). Co-treatment with 2 mM BSO and 20 mM NAC depleted GSH to a similar extent (P < 0.01) as 2 mM BSO, consistent with the function of BSO to inhibit the γ-GCS enzyme indispensable for GSH synthesis (Figure 3). This finding demonstrated that NAC suppressed DNA deletions in the BSO plus NAC treatment group (shown in Figure 2) directly and not by replenishing GSH pools. In 20 mM NAC treated mice, GSH levels increased by 30% (P < 0.05) (Figure 3) and the frequency of DNA deletions was slightly lower (10%) compared to untreated mice (shown in Figure 2), although the difference was not statistically significant. Apparently, an increase in GSH level above the normal level does not reduce the spontaneous frequency of rearrangements.

The levels of oxidized forms of GSH and GSSG were also measured. GSSG levels were extremely low (>200-fold) irrespective of the treatment scenario.

Cysteine concentration in BSO treated mice
Oxidative stress caused by GSH depletion may affect the status of other antioxidants. Therefore, we determined the levels of another relevant thiol, cysteine. Like GSH, cysteine levels dropped following BSO treatment. Cysteine levels were reduced by 27% (P < 0.05) and 55% (P < 0.01) in the mouse fetuses treated with 2 mM and 20 mM BSO, respectively, compared with untreated mice (Figure 4). On the contrary, the level of cysteine in co-treated mice with 2 mM BSO and 20 mM NAC was increased by 84% compared with 2 mM BSO treated mice (P < 0.001) and by 57% compared with untreated mice (P < 0.01). The increase in cysteine most probably originated from NAC (NAC itself was not detected), because NAC is readily deacetylated in cells to yield cysteine (19,20). The cysteine was not converted into GSH because of BSO-induced inhibition of γ-GCS (Figure 3). Apparently NAC prevented DNA deletions caused by GSH depletion (shown in Figure 2) by increasing the level of cysteine. Mice treated with 20 mM NAC had similar levels of cysteine as control mice, although higher GSH concentrations (shown in Figure 3) demonstrated that NAC can enhance the synthesis of GSH provided active γ-GCS enzyme is present.

The levels of oxidized form of cysteine, cystine, ranged from 0.051 to 0.067 mg/mg protein; no significant differences were observed between the groups.

Discussion
In this study we describe the effect of oxidative stress on large-scale genome rearrangements in a developing mouse. Oxidative stress, which was generated by treatment with a specific GSH synthesis inhibitor BSO, resulted in depletion of major thiol antioxidants, GSH and cysteine, and markedly elevated the frequency of 70 kb DNA deletions. This demonstrates that a decrease in the thiol antioxidant concentration in vivo causes irreversible genetic alterations. Such alterations can predispose...
individuals to cancer or other genetic disease. For example, DNA deletions can cause a loss of relevant genes including tumor suppressors, cell cycle regulators and DNA repair genes. It has not been previously shown that depletion of natural antioxidants can cause irreversible genome damage associated with large genome rearrangements. In accordance with our findings, GSH depletion has previously been found to be associated with elevated levels of 8-OhdG and DNA single strand breaks (SSBs) in cell culture (39,40), and chromatid breaks and sister chromatid exchanges in vivo (41). These effects can be attributable to mitochondrial destruction and release of large amounts of ROS (42) that occurs in tissues of GSH depleted rodent animals (14,16,18).

A cysteine analog NAC protected against DNA deletions in mouse fetuses depleted of GSH by BSO. NAC acts as GSH precursor and/or directly as an antioxidant (19,20,43). Metabolism of NAC to GSH requires γ-GCS, the very enzyme that is inhibited by BSO (10). In agreement with this, NAC did not replenish GSH in NAC plus BSO treated mice. On the other hand, NAC increased cysteine concentration by >80% compared with BSO treated mice and 50% compared with untreated mice. Thus, NAC apparently compensated for the decreased GSH level by increasing cysteine pools, which counteracted ROS and thereby suppressed DNA deletions. A protective effect of NAC was expected, because a number of studies demonstrated that NAC is able to reduce or counteract oxidative damage. It is of particular relevance that NAC prevents damage to DNA in a developing mouse. For example, NAC suppresses formation of 8-OhdG DNA lesions and bulky DNA adducts resulting from oxidative stress naturally occurring at birth (22) or following exposure to cigarette smoke (23). Additionally, NAC suppresses 8-OhdG and DNA deletion formation in Atm deficient mice that exhibit chronic oxidative stress and damage spontaneously (30). Some studies showed that NAC concurrently given with BSO reduced the effect of GSH depletion on enhancing drug toxicity to varying degrees. For example, NAC reduced DNA damage (measured with the comet assay) in UV irradiated lung fibroblasts depleted of GSH by BSO (44). In another study, NAC rescued cells from toxic effects induced by dopamine and enhanced by BSO (45). On the contrary, NAC co-treatment with BSO was not preventive against endothelial cell injury caused by sulfur mustard (46) or against lipid peroxidation in hyperoxic conditions in a rat model (47).

As discussed before (30), oxidative stress appears to be especially hazardous in growing tissues, because only in replicating cells oxidative genome lesions, such as 8-OhdG, SSBs (48–50) or stalled replication forks (51,52), can lead to genetic recombination and result in permanent genome rearrangements. For example, during replication unrepaired 8-OhdG residues cause SSBs, which produce highly recombining double strand breaks (DSBs) when the broken site on the template is reached. Arrested DNA replication by stalled replication forks is re-initiated through homologous recombination (53,54), which can lead to deletions or translocations when it occurs between repetitive elements. Findings using a similar DNA deletion assay in yeast support that DNA replication is required for formation of DNA deletions induced by various DNA lesions, including SSBs (55,56). In general, embryonic cells are characterized by a high replication index and, therefore, might be particularly susceptible to oxidative genome damage. The tissue examined in this study, the RPE, is a monolayer of epithelial cells consisting of only 54,000 cells (35). The RPE development begins at Embryonic-day 8, reaches its maximal growth of ~4000 cells per day in the middle of gestation and ceases within the first two weeks after birth (35,59).

In summary, our study showed that oxidative stress generated by thiol depletion leads to DNA deletions during mouse development. Thus, it is possible that oxidative stress may play a role in carcinogenesis by causing DNA deletions or other irreversible genome rearrangements. Proliferating tissues that are inherently more susceptible to cancer (57,58) may be at the highest risk.

Acknowledgements

We thank Mitsuko Lynn Yamamoto for comments on the manuscript, and Nicholas Carls and Elvira Fischer for technical help. This study was supported by grants from the National Institute of Environmental Health Sciences, NIH (RO1 grant No. ES09519 to RHS), a post-doctoral research fellowship of the University of California Toxic Substances Research and Teaching Program, and the Lymphoma Research Foundation Elizabeth Banks Jacobs & Byron Wade Strunk Memorial Fellowship (both to R.R.).

Conflict of Interest Statement: None declared.

References