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Oxidative DNA damage in cultured fibroblasts from patients with hereditary glutathione synthetase deficiency

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Abstract
The SH compound glutathione (GSH) is involved in several fundamental functions in the cell, including protection against reactive oxygen species (ROS). Here, we studied the effect on oxidative DNA damage in cultured skin fibroblasts from patients with hereditary GSH synthetase deficiency. Our hypothesis was that GSH-deficient cells are more prone to DNA damage than control cells. Single cell gel electrophoresis (the comet assay) in combination with the formamidopyrimidine DNA glycosylase enzyme, which recognizes oxidative base modifications, was used on cultured fibroblasts from 11 patients with GSH synthetase deficiency and five control subjects. Contrary to this hypothesis, we found no significant difference in background levels of DNA damage between cells from patients and control subjects. To study the induction of oxidative DNA damage without simultaneous DNA repair, the cells were γ-irradiated on ice and DNA single-strand breaks measured. The patient and control cells were equally sensitive to induction of single strand breaks by γ-irradiation. Therefore, factors other than GSH protect DNA from oxidative damage. However, cells with a high background level of oxidative DNA damage were found to be more sensitive to ionizing radiation. This suggests that differences in background levels of oxidative DNA damage may depend on the cells' intrinsic protection against induction of oxidative damage.

Keywords: *Glutathione, glutathione synthetase, glutathione synthetase deficiency, comet assay, DNA damage, oxidative stress*

Abbreviations: *8-oxo-dG, 8-Hydroxy-2′-deoxyguanosine; BSA, Bovine serum albumin; CNS, Central nervous system; EDTA, Ethylenediaminetetraacetic acid; FPG, Formamidopyrimidine DNA N-glycosylase; GSH, Glutathione; L-CPA, L-2-chloropropionate; MEM, Minimal essential medium; OMIM, Online mendelian inheritance in man; PBS, Phosphate buffered saline; ROS, Reactive oxygen species; SH compound, Sulfhydryl compound; TM, Tail moment*

Introduction

Oxygen, although vital for life, is also toxic. Incomplete reduction of oxygen to water generates highly toxic radicals, collectively termed reactive oxygen species (ROS). About 2–4% of the total oxygen consumed during electron transport is converted to ROS [1]. ROS can readily react with DNA, proteins and lipids to cause mutations, protein destruction and peroxidation of membrane lipids. Excessive production of ROS may be involved in the pathogenesis of several diseases such as cancer, atherosclerosis and neurodegenerative diseases [2–4]. Moreover, ROS are thought to be involved in ageing [4,5]. It has been estimated that about 10,000 oxidative DNA lesions are produced per human cell each day [6]. Oxidative DNA lesions include structural damage (i.e. strand breaks) and/or modification of the bases. 8-Hydroxy-2′-deoxyguanosine (8-oxo-dG) is one

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of the commonest lesions formed from the reaction of oxyradicals with DNA [7]. The 8-oxo-dG accumulates in tissues, including the brain, during ageing [8].

Excessive production of ROS induces cellular damage that causes apoptosis or necrosis and genotoxic effects. The cells are however, protected against ROS induced damage by specific metabolizing and scavenging systems. Mammalian cells contain several compounds, which may act as antioxidants and free radical scavengers. The tripeptide GSH is the principal intracellular non-protein SH compound. It is found in most mammalian cells at concentrations up to 10 mM [9]. Glutathione takes part in several fundamental biological functions, including handling of ROS, detoxification of xenobiotics and carcinogens, redox reactions, biosynthesis of DNA and leukotrienes and neurotransmission. GSH and other SH compounds tend to react with free radicals, such as the OH radical, but may also chemically restore damage to macromolecules by donation of a hydrogen atom. This mechanism is believed to be essential for the protective effect of hypoxia during irradiation [10].

Human GSH synthetase is a homodimer with a subunit size of 52 kDa. The three dimensional structure of the GSH synthetase enzyme has been determined [11]. The gene, located on chromosome 20q11.2, consists of 13 exons [12,13]. *Glutathione synthetase deficiency* (OMIM #266130) has been confirmed in more than 65 patients in about 55 families. About 25% of all patients with this condition have died in childhood—often in the neonatal period—due to electrolyte imbalance and infections. The disease phenotype is associated with a wide range of clinical findings from a mild disease with only hemolytic anemia to a severe disease with hemolytic anemia, metabolic acidosis, 5-oxoprolinuria and progressive neurological symptoms, including mental retardation, seizures, spasticity and cerebellar symptoms, e.g. ataxia. A thorny question is to determine the pathogenesis of the progressive neurological symptoms, which has not been resolved. In a long-term follow-up study of 28 patients with GSH synthetase deficiency, it was shown that the antioxidants vitamin E and C seemed to protect the CNS against damage [14]. The nervous system is especially vulnerable to oxidative injury because of a high rate of oxidative metabolic activity, and a high concentration of readily oxidizable substrates, in particular, polyunsaturated fatty acids and a low level of protective antioxidant enzymes [15].

In the present paper, we compared cultured skin fibroblasts from patients with GSH deficiency and control subjects as regards the background levels of oxidative damage and sensitivity of DNA to an oxidative agent (ionizing radiation). To measure oxidative DNA damage, we used the FPG enzyme that converts oxidized purines and alkali-labile abasic sites to strand breaks. DNA strand breaks were then determined by alkaline single cell gel electrophoresis (the comet assay).

Materials and methods

Patients

Eleven patients with GSH synthetase deficiency from Europe, Asia and the Middle East were investigated. Six were females and five males. Five patients had parents who were known to be consanguineous. Nine patients were homozygous, one heterozygous and one compound heterozygous for a mutation in the glutathione synthetase gene. One patient had died at 3 weeks of age. Among the survivors, the age at last follow-up ranged from 2 to 32 years (median 10 years). In all patients, the diagnosis of GSH synthetase deficiency was based on finding a marked reduction in GSH synthetase activity in cultured fibroblasts or erythrocytes. In addition, a decrease in GSH in erythrocytes and massive 5-oxoprolinuria (pyroglutamic aciduria) as well as metabolic acidosis, hemolytic anemia and neurological symptoms were findings supporting the diagnosis. Two patients had mild, five moderate and four severe disease. The treatment varied among the patients. It included correction of acidosis, and supplements of vitamin C and/or E. The age of the patients when the skin biopsy was taken ranged from 3 weeks to 9 years (median 6 months). The cultured patient cells were in passage 5–15 (median 9). The studies were approved by the Ethics Committee of Karolinska Institutet, Stockholm, Sweden.

Control subjects

Control subjects were obtained, after informed consent, among metabolically healthy children undergoing minor surgery, such as correction of an inguinal hernia/umbilical hernia or polydactylia. The age of the control subjects when the skin biopsy was taken ranged from 1 to 15 (median 4) years. The cultured control cells were in passage 6–10 (median 9).

Preparation of cultured fibroblasts

Fibroblasts were obtained by skin biopsies of patients and controls. Cultured fibroblasts were grown in MEM culture medium containing 10% fetal bovine serum, penicillin and streptomycin. The cells were harvested in the late log phase of growth by means of trypsin digestion, washed three times with phosphate-buffered saline solution, frozen and stored at −70°C, pending analysis. All cell cultures were found to be free of mycoplasma.

GSH synthetase activity

We measured GSH synthetase in erythrocytes and fibroblasts as described by Ristoff et al. in 2001 [14] and protein with a commercial assay (Bio Rad, Protein Assay).

Irradiation

The cells were trypsinized and irradiated with 0, 3 and 6 Gy, respectively, on ice in PBS in a ^{137}Cs gamma ray source at 4.3 Gy/min.

Detection of DNA damage (comet assay)

The comet assay was essentially that of Olive et al. [16] with the modifications described by Yang et al. [17]. We used Teflon-coated slides with three Teflon-free areas each measuring 14 mm in diameter (Histolab AB, Goethenburgh, Sweden). A measure of 30 μl of 0.75% low melting point agarose in PBS kept at 37°C was mixed with 4 μl of cell suspension ($0.5-1 \times 10^6$ cells/ml) and spread over the slides. The slides were left to solidify on an ice tray before immersing them for an hour in an ice-cold freshly made lysing solution (2 M NaCl, 25 mM EDTA, 20 mM Tris, pH 10 and 0.5% Triton X-100). They were kept on ice for 1 h in 25 mM EDTA in PBS and were then transferred for 1 h to an ice-cold enzyme buffer, 20 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5 and 0.1 mg/ml BSA. They were thereafter put on a chilled plate and 20 μl of formamidopyrimidine-DNA glycosylase (FPG) protein (Trevigen Inc., Gaithersburg, MD, USA) (1 μg/ml) or enzyme buffer was spread over the slides. The slides were incubated on an ice tray for 30 min for the diffusion of enzyme into the gels, and in a humidified chamber at 37°C for 60 min to allow enzymatic conversion of oxidized bases to single-strand breaks. They were then treated with 30 mM NaOH, 1 M NaCl, 1 mM EDTA and 0.5% of the detergent sodium N-lauryl sarcosinate for 1 h and 30 mM NaOH and 1 mM EDTA for 45 min. Electrophoresis was done at room temperature, in darkness, in a Bio-Rad subcell GT unit containing the same solution, for 15 min using 20 V (0.67 V/cm). After electrophoresis, the slides were neutralized, rinsed, air-dried, fixed in methanol and stored pending analysis. Ethidium bromide was used to stain the DNA and the comets were examined in a fluorescence microscope, using the program Komet II from Kinetic Imaging. Images of 50 randomly-selected cells were analyzed from each sample and the tail moment (TM) determined with Olive's method [18]. Oxidative damage was determined as FPG-sensitive sites, i.e. the difference in tail moment between cells incubated with or without the FPG enzyme. The level of background single-strand breaks, TM approximately 7, was not different in cells from patients and controls.

Statistical methods

Data were expressed as median and range. Analysis for significance of a difference was done with Student's *t*-test. *P*-values < 0.05 were considered significant. The correlation between background damage and gamma ray induced DNA strand breaks was tested using the least squares fit line and the Spearman rank correlation test.

Results

The data concerning the patients and control subjects with respect to levels of SH-compounds, enzyme activity, DNA damage as well as data on age at donation of cells, severity of clinical symptoms etc. are summarized in Table I. To measure oxidative DNA damage, we used the FPG enzyme which converts certain oxidized purines, such as 8-oxo-dG, to strand breaks [19]. Tail moment was preferred to % DNA in tail as the latter showed a tendency to saturate at 6 Gy. For comparison the % DNA in the tail are summarized in Table II. As seen in Table I, no increase in background levels of oxidative DNA damage, measured as FPG-sensitive sites, was found in fibroblasts from the GSH-deficient patients. The values of the levels of FPG-sensitive sites are higher in this study than reported elsewhere [20]. This may be due to the fact that most other studies use 0.3 M NaOH, which also detect alkali-labile sites; they will therefore, be seen as part of the background of strand breaks. Our alkaline conditions (30 mM NaOH) will not convert these alkali-labile sites to breaks, but FPG will do so, and so our FPG-sensitive sites include an unknown number of alkali-labile damage, such as abasic sites as well as oxidised bases. Also, the calibration with ionising radiation will be influenced by the concentration of NaOH. The damage had a normal distribution, i.e. no sub-populations with higher or lower levels of damage were present (data not shown). The background level of damage is a steady state determined by induction and repair of damage. To study the induction of DNA damage at a certain level of oxidative challenge, we irradiated the cells with γ-rays and measured DNA strand breaks. Strand break induction after irradiation on ice will reflect action of GSH as a radical scavenger but not (to a large extent) the function of GSH as co-factor for glutathione peroxidase. No evidence of an increase in the rate of induction of single-strand breaks by radiation in the deficient cells was seen (Figure 1). In Figure 2, the correlation between FPG-sensitive sites and levels of the intracellular SH compounds (GSH, γ-glutamyl cysteine and cysteine) have been plotted. We found no significant decrease in DNA damage with an increase in SH levels. No correlation was seen between SH levels and the slopes of the γ-ray induction curves for the various cell lines (data not shown). Further, there was no correlation

Table I. Levels of GSH and SH compounds, activity of GSHsynthetase, FPG-sensitive sites and sensitivity for radiation induced single-strand breaks in fibroblasts from patients with GSH synthetase deficiency and controls.

Patient GS number/sex	Passages	Age when sample was taken	Severity of disease	GSH* (nmol/mg protein)	GS-activity† (pkatal/mg protein)	GSH+γ-glut+cystein*	Net. FPG-sensitive sites (TM)	Radio senstivity (TM/Gy)
34/F	9p	Before 7 years	Mild	ND	0.04	ND	20.5	ND
47/M	e8p	7 month	Moderate	6.44	1	30.1	26.6	4.1
40/M	e6p	9 years	Severe	11.46	4.4	50.5	29.4	4. 4
46/F	15p	1 year	moderate	11.2	8.1	52.2	26.1	4.7
2/F	5p	Before 13 year	moderate	ND	0.09	ND	32.1	5.6
29/M	9p	Before 1 year	severe	ND	4.8	ND	29.9	4.1
48/M	9p	5 month	moderate	7.18	3.0	37.2	30.48	3.8
41/F	e14p	5 month	severe	11.93	1.8	28.2	45.4	4.8
23/F	9p	3 weeks	moderate	7.38	5.4	132	34.3	5.0
20/F	9p	Before 5 months	Mild	2.84	1.6	49.2	45.8	6.6
33/M	15p	Before 4 months	severe	ND	1.27	ND	42.8	5.0
Controls								
1/F	6p	1 year		51.4	45.6	62.8	26.7	3.9
2/M	10p	2 years		ND	35.2	ND	40.1	4.6
3/F	9p	15 years		27.9	ND	37.4	42.1	5.3
4/F	9p	4 years		38.3	43.8	50.8	34.8	6.6
5/M	10p	13 years		ND	33.9	ND	43.5	4.8

"e" means that the cell cultures have grown undefined number of passages in an external laboratory before arrival to our laboratory. The number indicates the number of passages in our laboratory; ND means not determined.* From Ristoff et al. [26].† From Ristoff et al. [14] and [26].

between the background levels of oxidative DNA damage or the rate of strand break induction by gamma rays and sex, age, treatment, severity of symptoms or the number of passages of the cell cultures. However, there was a significant correlation ($r^2 = 0.265$, $P < 0.05$, Spearmans rank correlation test $P < 0.02$) between the induction slopes and the background levels of damage (Figure 3).

Discussion

Various cell populations may have higher background levels of damage than others because of lower intracellular protection against oxidative damage induction or repair of damage. The intrinsic production of ROS may also be increased. The latter is probably true, for example, in patients with diabetes in whom not only DNA damage has been reported to increase (e.g. [21]) but also lipid peroxidation (e.g. [22]). Some data suggest that ROS may be involved in the pathogenesis of GSH synthetase deficiency. Pathological autopsy findings have been found in the CNS, with selective atrophy of the granule cell layer of the cerebellum in two patients with GSH synthetase deficiency ([23]; personal correspondence Dr M. Pronica, Department of Pathology, Children's Memorial Health Institute, Warsaw, Poland). In rats,

Table II. Percent DNA in tail for different treatments.

	Patients median (range)	Controls median (range)
0 Gy	11.2 (7.4–14.2)	9.1 (6.6–13.7)
3 Gy	53.5 (48.1–60.0)	53.8 (48.2–56.4)
6 Gy	76.8 (73.5–82.3)	78.9 (71.3–83.9)
Buffer	13.8 (8.5–18.7)	17.0 (9.4–20.3)
FPG enzyme	61.7 (48.2–68.5)	66.5 (53.4–71.3)

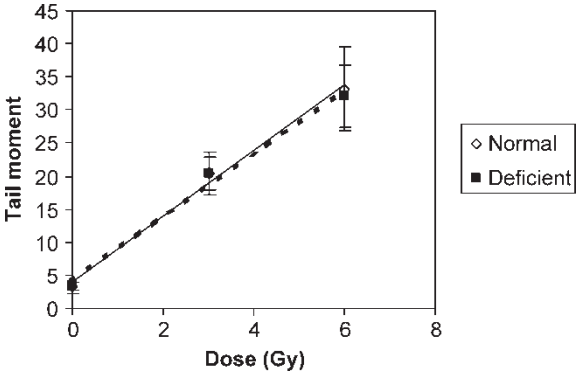

Figure 1. Induction of DNA strand breaks by gamma irradiation in normal and glutathione-deficient cells. Average ± 1 SD from 5 (control) or 10 (deficient) donors.

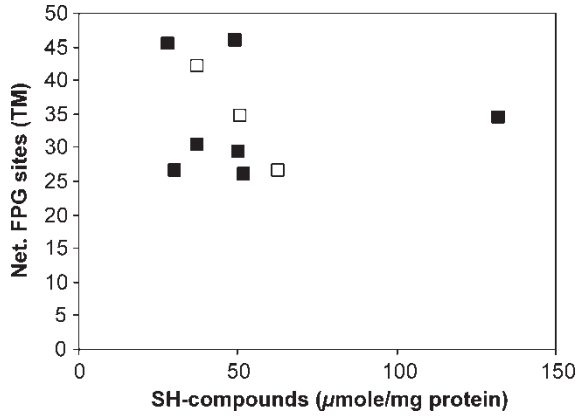

Figure 2. Number of FPG-sensitive sites as a function of the concentration of SH compounds, measured as the sum of GSH, γ-glutamylcysteine and cysteine. The data in 3 (open symbols) controls and 7 patients (filled symbols) are shown. The reference values for thiol concentration were obtained from Ristoff et al. [26]. One cell line was (in repeated measurements) considerably higher in the levels of SH-compounds compared to the other. There was no significant correlation between FPG-sensitive sites and SH-compounds even if this data point was omitted.

oral treatment with L-2-chloropropionate (L-CPA)—an intermediate in the production of chemical agricultural products, which stimulates the formation of ROS—causes selective necrosis in the granule cell layer of the cerebellum *in vivo* and in cultured rat cerebellar granule cells *in vitro* [24]. It has also been shown that L-CPA reduces the level of GSH in the brain, and that the granular cell lesion is increased by inhibiting GSH synthetase experimentally [25]. The antioxidant vitamin E protects against L-CPA-induced cell death [24]. In patients with GSH synthetase deficiency, the antioxidants vitamin E and C also seem to protect the CNS against damage [14]. There is thus a connection between ROS, cerebellar granule cells and GSH

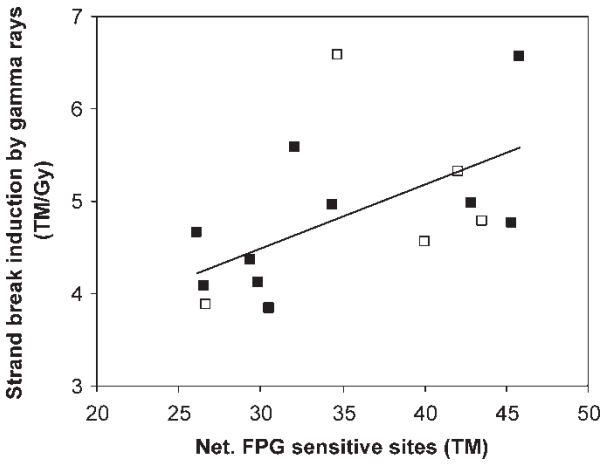

Figure 3. FPG-sensitive sites as a function of the slope of the induction curves for DNA strand breaks induced by γ-radiation. The data in 5 controls (open symbols) and 10 patients (filled symbols) are shown.

although there is no direct proof that a causative relationship exists. One hypothesis that may explain the progressive neurological symptoms in patients with GSH synthetase deficiency could be that deficiency of GSH makes cells more prone to oxidative damage of DNA and other cellular macromolecules, which leads to apoptosis and neuronal death.

In this study, the finding that background oxidative damage correlated with γ-ray induction slopes (Figure 3) indicates that the level of background damage to some extent, depends on protection against induction of oxidative DNA damage. With this model, we found no evidence that GSH itself is essential to the protection of DNA against oxidative damage. However, it has recently been shown in 7 of the 11 cell lines studied that the total levels of the non-protein SH compounds, i.e. GSH, γ-glutamylcysteine and cysteine—are about the same in GSH-deficient and control cells [26]. Especially, the levels of γ-glutamylcysteine increase markedly in the fibroblasts of patients. We speculated that as γ-glutamylcysteine contains both reactive moieties of GSH, i.e. the γ-glutamyl and sulfhydryl residues—it might replace GSH in certain functions. The γ-glutamylcysteine may therefore, at least partly, replace GSH in the cellular defense against oxidative stress and thereby prevent the serious consequences of insufficient GSH levels. Also, no significant correlation was found when background oxidative damage was plotted against the sum of the levels of GSH, γ-glutamylcysteine and cysteine (Figure 2). One outlier had three times the median levels of SH-compounds. The measurement was repeated with similar result. There was no significant correlation between the level of SH-compounds and background damage even if this outlier was omitted. Nevertheless, SH compounds may have an important protective effect against oxidative DNA damage, but an increase in concentration over a certain level will not increase the protection further. The SH compounds may also be of greater importance in more hypoxic environments, such as tissues *in vivo*, but the experiments were done in cell cultures that have higher oxygenation. Previous studies have also shown that the chromatin structure with DNA-bound proteins may be of more value for intracellular protection of DNA against radical-mediated damage than soluble scavengers [27–29].

Many publications have discussed the effect of GSH-depletion by buthionine sulfoximine (BSO). DNA damage has been shown to increase after treatment with BSO after challenges with ionising radiation [30], UV light [31] and chemicals [e.g 32] but increased damage has also been reported for BSO treatment alone [33]. This substance inhibits the synthesis of γ-glutamylcysteine and depletes cells of total levels of SH-compounds. This is in contrast to the cells used here where the total levels of SH-compounds are similar and only the relative contribution of GSH and γ-glutamylcysteine differs.

Steady state levels of DNA damage are the result of the rate of endogenous damage induction, on the one hand, and repair of damage, on the other. It is thus not possible to say for certain if there is any difference in induction between GSH deficient and normal cells just from the background values, as increased repair could obscure an increased induction. To assess the rate of induction of oxidative DNA damage without interference of repair we used ionizing radiation. About 2/3 of the single-strand breaks induced by gamma irradiation are formed indirectly from OH radicals by ionization of water [34]. We again found no difference between cell cultures from GSH-deficient patients and controls. There was no correlation between the levels of SH compounds and the rate of induction of damage by radiation (data not shown). This suggests that differences in the levels of SH compounds as large as in this study do not play an essential role in protection against the induction of DNA damage by ionizing radiation. However, the results should not be interpreted as evidence that SH compounds do not play a role in protection against DNA damage in general, only that the variations in the levels of SH-compounds found in the cells used here are not correlated to the protection against oxidative DNA damage. Also, the median total levels of SH compounds did not differ between patients and controls. The GSH deficiency seems to be compensated by an increase in other SH-compounds in these patients.

It is difficult to measure oxidative DNA damage accurately because oxidation of DNA may occur during laboratory analyses of the samples although the comet assay with damage specific enzymes seems to be less affected by this problem than chromatographic assays [20]. However, the correlation between background damage and induction of damage by γ-irradiation in this study shows that the method used here is not greatly affected by such oxidation.

Our findings suggest—contrary to the original hypothesis—that the mechanisms underlying the severe clinical symptoms associated with GSH synthetase deficiency do not involve oxidative damage of nuclear DNA, at least not where fibroblasts are concerned. Oxidation of other cellular compounds, a change in the redox potential in the cell, a difference in biochemical activity of the GSH and γ-glutamylcysteine or toxic effects of GSH precursors may be more likely explanations. The deficiency in neurotransmitter/modulator function of GSH may also account for the progressive neurological symptoms seen in these patients.

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