GSH loss *per se* does not affect neuroblastoma survival and is not genotoxic

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Abstract. Depletion of glutathione (GSH) by buthionine sulfoximine (BSO) has been reported to be toxic against some cancer cells and to sensitize many tumours including neuroblastoma (NB) to anticancer drugs. The balance between the production rate of reactive oxygen species (ROS) and the function of GSH affects the intracellular reduction-oxidation status, which is crucial for the regulation of several cellular physiological functions. To assess the role of glutathione in neuroblastoma therapy, the effect of sublethal concentrations of BSO was studied in a panel of neuroblastoma cell lines characterized by different MYCN status. We found that GSH depletion *per se* not accompanied by ROS overproduction, does not affect cell survival, and is not genotoxic but induces HO-1 expression in GI-ME-N cell line, a representative example of MYCN non-amplified NB cells, having the highest basal levels of GSH among the tested NB lines. These observations might open a novel therapeutic window based on the possibility of modulating the cellular 'activity' of GSH.

Introduction

Cancer cells are, in general, under constant oxidative stress because of their abnormal metabolic activity (1) and are vulnerable to further oxidative stress, suggesting that changes in cellular redox status are a possible anticancer strategy. Reactive oxygen species (ROS) have been considered as DNA damaging agents that increase the mutation rate and promote oncogenic transformation (2). However, in contrast with their pathological role, intracellular ROS can act as second messenger molecules in signalling cascades, controlling diverse cellular events such as proliferation, apoptosis and inflammation (3-5).

The balance between the rate of ROS production and the function of antioxidants, such as glutathione (GSH), affects the intracellular reduction-oxidation status, which is crucial for the regulation of several cellular physiological functions.

GSH is a multifunctional molecule involved in the maintenance of cellular homeostasis, and because of the cysteine residue, is readily oxidized non-enzymatically to glutathione disulfide (GSSG) by electrophilic substances (6-8).

Buthionine sulfoximine (BSO) which irreversibly inhibits γ-glutamylcysteine synthetase (GCS), the rate-limiting step in the synthesis of GSH (8), has been shown, *in vitro*, to sensitize tumoural cells to several anti-cancer drugs (9-13).

BSO-induced GSH depletion has been demonstrated to increase oxidative stress and to be toxic in some malignancies, including acute leukemia and neuroblastoma (14,15). Neuroblastoma is the most common solid tumour in childhood and shows remarkable biological heterogeneity (16). The adverse prognosis of neuroblastoma is largely associated with the amplification of the MYCN oncogene (17), which determines the inability of neuroblastoma to trigger apoptotic death induced by tumour necrosis factor-related apoptosis inducing ligand (TRAIL) system (18). Neuroblastoma cell survival is strictly related to availability of antioxidants such as GSH and its apoptotic death might be triggered by modulating intracellular thiol levels (9,15).

Recently, it has been found that GSH depletion is associated with elevated levels of 8-OH deoxyguanosine (8-OHdG) and DNA single strand breaks in cell culture (19); moreover, it induces DNA deletions in mice (20).

When ROS overproduction following BSO exposure exceeds the antioxidant protective actions of heme oxygenase-1 (HO-1), mitochondrial membrane damage occurs, leading to apoptosis and/or necrosis (21,22). HO-1 induction by stress related agents in several types of human cancer cells has been reported to play a role in chemoresistance to apoptosis (23,24).

To investigate the potential role of GSH loss-oriented therapy in neuroblastoma, we studied the *in vitro* toxicity of different BSO concentrations (1-100 μM) against neuroblastoma cell lines displaying different MYCN status.

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Materials and methods

Cell cultures and treatments. Our study was carried out in five human neuroblastoma cell lines with different MYCN status: ACN, SH-SY-5Y and GI-ME-N cell lines without MYCN amplification and two MYCN-amplified cell lines, SK-N-BE-2(C) and LAN5. All neuroblastoma cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% penicillin/streptomycin, 1% sodium piruvate, 1% of non-essential amino acid solution and 1% antimycotic solution. Cells were incubated at 37˚C in humidified atmosphere of 95% air and 5% CO_2 and were split and seeded in new flasks (75 cm^2) every two days to maintain them in log-phase. Cells ($10x10^6$/flask) were treated with concentrations 1, 10, 50, 100 μM of L-buthionine-S,R-sulfoximine (BSO) for 24 h. Moreover, SK-N-BE-2(C) and GI-ME-N cells were exposed for 1, 3, 6, 12 and 24 h to 10 μM L-BSO. All chemical reagents cited in this subsection were from Sigma Chemical Co. (St. Louis, MO, USA).

Assay of GSH levels in HPLC. GSH content was evaluated by HPLC (25). Briefly, specimens for the evaluation of total GSH (tGSH) were harvested in PBS and precipitated with perchloric acid (PCA) (10% final); thiol groups were blocked with iodoacetic acid (IAA) at alkaline pH; the analytes were then converted to 2,4-dinitrophenyl derivatives with 1% 1-Fluoro-2,4-dinitrobenzene (FDNB) at 4˚C in the dark overnight.

Quantitative determination of derivatized analytes was performed in HPLC; the HPLC system was equipped with an NH_2 Spherisorb column and a UV detector set at 365 nm; the flow rate was 1.5 ml/min. The mobile phase was maintained at 80% A (80% methanol) and 20% B (0.5 M sodium acetate in 64% methanol) for 5 min, followed by a 5-min linear gradient to 1% A and 99% B; the mobile phase was maintained at 99% B for 15 min. Total GSH content was evaluated in the chromatograms as GSH+2GSSG, and expressed in GSH equivalents (26). GSSG/tGSH ratio is calculated as nmol GSSG/nEq tGSH. Data were obtained from 3-4 experiments for each cell line; each experiment was performed in duplicate or triplicate; the final data are expressed as mean ± SEM.

Measurement of reactive oxygen species (ROS) production. Neuroblastoma cells were grown on chamber slides (Iwaki Seiyaku Co., Tokyo, Japan), subjected to BSO treatments for the indicated times and then incubated for 20 min at 37˚C with 20 μM 2'-7' dichlorofluorescein-diacetate (DCFH-DA, Sigma), a cell-permeable, non-fluorescent precursor of DCF that can be used as an intracellular probe for oxidative stress (27,28). Accumulation of DCF in cells was measured by an increase in fluorescence at 530 nm when the sample was excited at 485 nm. The cells were observed and counted (5 fields of ~10^2 cells each) using a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) with a standard set of filters for fluorescein.

Annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. This assay is based on the ability of Annexin-V to bind to the phosphatidylserine exposed on the surface of cells undergoing apoptosis and the capacity of propidium iodide to enter cells that have lost their membrane integrity (29). Neuroblastoma cells were grown and treated on chamber slides (Iwaki Seiyaku Co.), the medium was discarded and the cells were incubated in the dark for 5 min at room temperature with 200 μl of 1X binding buffer, 0.5 μg/ml of FITC-labelled recombinant Annexin-V and 0.5 μg/ml of propidium iodide (BioVision, Mountain View, CA, USA). The cells were observed and counted (5 fields of about sixty cells each) under a fluorescence microscope (Leica DMIRB) using a dual filter set for FITC and rhodamine. To evaluate apoptotic phenomena, we considered the percentage of Annexin-V positive/propidium iodide negative cells.

Single cell gel electrophoresis (comet assay). Formamidopyrimidine DNA glycosylase (Fpg)-modified comet assay (30) was used to evaluate DNA oxidative damage. This test employs the Fpg enzyme, a glycosylase that recognizes and specifically cuts the oxidized bases, principally 8-oxoguanine, from DNA, and so produces apurinic sites, which are then converted in breaks by the associated AP-endonuclease activity. The procedure of Tice and Strauss (31) was followed with minor modifications. The comet assay protocol was carried out under dim light to prevent any additional DNA damage. After treatments, cells were trypsinized and cell suspensions ($15x10^3$ cells) were mixed with low melting point agarose (0.5% in PBS) and spread on slides with a thin layer of normal melting point agarose (1.5% in PBS). The slides were washed three times in enzyme buffer (50 mM Na_3PO_4, 10 mM EDTA, 100 mM NaCl, pH 7.5), drained and incubated with 50 μl of either buffer or Fpg (1 μg/ml in enzyme buffer) in the dark for 30 min at 37˚C. The slides were placed in a horizontal gel box near the anode end, and covered with electrophoretic buffer (300 mM NaOH, 1 mM EDTA, pH 13.0); after 30 min, the slides were subjected to an electric field of 300 mA for 40 min. Finally, the slides were coated with neutralisation buffer (0.4 M Tris-HCl pH 7.5), dried and incubated for 10 min in absolute ethanol. The slides were finally stained with 50 μl of 20 μg/ml ethidium bromide staining solution, covered with a cover slip and analysed by means of a fluorescence microscope (Leica DIMRB) with an excitation filter of 515-560 nm and a barrier filter of 590 nm.

RNA isolation and RT-PCR analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions and quantified spectrophotometrically. Total RNA (1 μg) was reverse transcribed into cDNA by random hexamer priming and SuperScript™ II Reverse Transcriptase (Invitrogen).

Amplification of cDNA by polymerase chain reaction was performed using PCR Master Mix (Fermentas, Hanover, USA) and specific primers. GAPDH expression was used as housekeeping gene. The sequences of the primers were as follows: HO-1, forward GCT CAA CAT CCA GCT CTT TGA GG; reverse GAC AAA GTT CAT GGC CCT GGG A (amplicon length 284 bp); GAPDH, forward GTC TTC ACC ACC ATG GAG AA; reverse ATC CAC AGT CTT CTG GGT GG (amplicon length 266 bp).

Polymerase chain reaction products were separated by electrophoresis on a 1.5% agarose ethidium bromide gel and

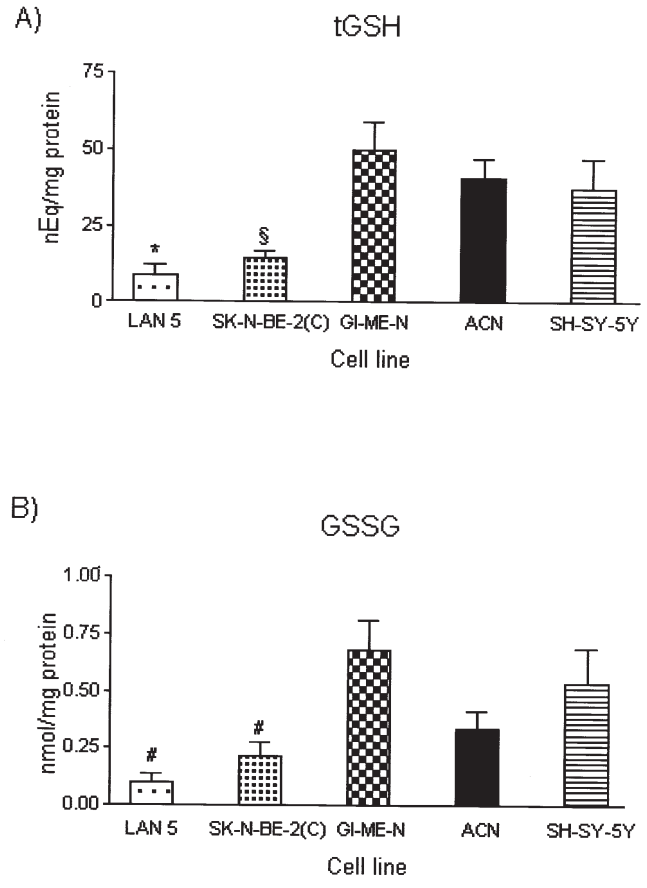

Figure 1. Content of total glutathione [tGSH, (A)] and oxidized glutathione [GSSG, (B)] in the indicated neuroblastoma cell lines. The concentrations of the analytes were determined by HPLC analysis. Data are expressed as mean ± SEM. ANOVA plus Newman-Keuls multiple comparison test: *p<0.05 vs GI-ME-N, ACN and SH-SY-5Y; §p<0.05 vs GI-ME-N and ACN; #p<0.05 vs GI-ME-N.

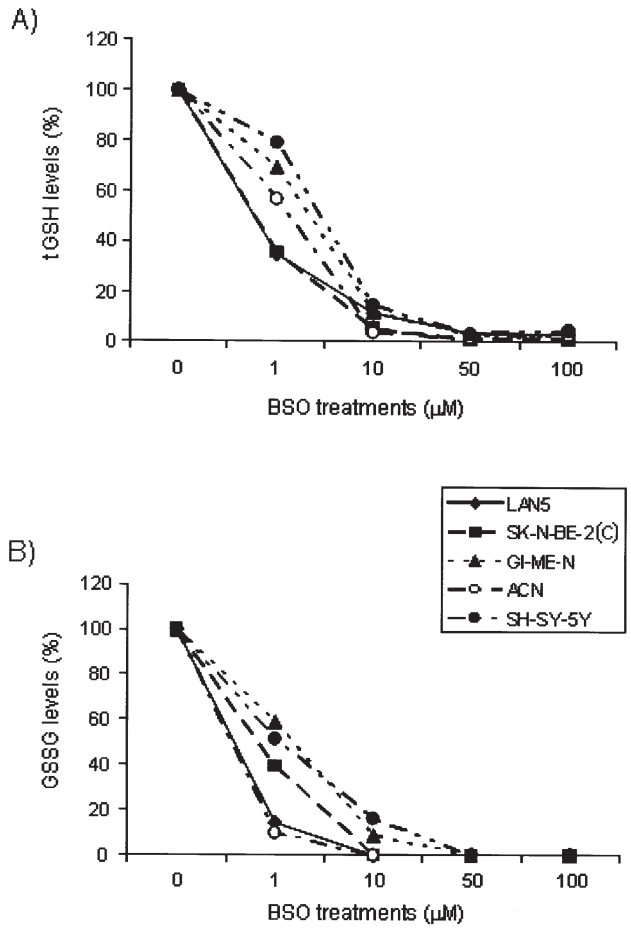

Figure 2. Changes in tGSH (A) and GSSG levels (B) in the indicated neuroblastoma cell lines exposed for 24 h to increasing concentrations (1-100 μM) of BSO. The concentrations of the analytes were determined by HPLC analysis and expressed as % of the control value.

analysed with the Gel Doc 2000 densitometer (Milan, Italy) through the 'Molecular Analyst' software (BioRad, Milan, Italy).

Statistical analysis. Results were expressed as mean ± SEM; from at least three independent experiments. The statistical significance of parametric differences among sets of experimental data was evaluated by One-way ANOVA and Newman-Keuls post-test for multiple comparisons.

Results

MYCN amplified cells show a lower amount of tGSH and GSSG than non-amplified cells. Intracellular content of tGSH has been evaluated by HPLC analysis; we found that MYCN amplified cell lines showed a lower amount of this thiol [LAN5: 8.37±3.35 and SK-N-BE-2(C): 14.17±2.66 nEq/mg of protein] in comparison with non-amplified cells (GI-ME-N: 49.92±9.34, ACN: 41.0±6.26; and SH-SY-5Y: 37.23±9.78 nEq/mg of protein; Fig. 1A). ANOVA indicated highly significant difference (p=0.032); Newman-Keuls post-test indicated significant difference between LAN5 and all non-amplified cell lines, and between SK-N-BE-2(C) and all non-amplified cells except SH-SY-5Y, probably due to the relatively high variance of the tGSH level in the latter.

Intracellular GSSG evaluation gave a similar pattern of results (Fig. 1B); globally, MYCN amplified cell lines had lower GSSG levels [LAN5: 0.102±0.037 and SK-N-BE-2(C): 0.216±0.057 nmol/mg of protein] than non-amplified ones (GI-ME-N: 0.677±0.135, ACN: 0.336±0.079 and SH-SY-5Y: 0.533±0.156 nmol/mg of protein). ANOVA indicated highly significant difference (p=0.0148); Newman-Keuls post-test indicated significant difference between LAN5 and GI-ME-N and between SK-N-BE-2(C) and GI-ME-N. The content in GSSG of ACN was lower but not significantly different from the other MYCN non-amplified cell lines.

The ratio between GSSG and tGSH remained <2% in each cell line. ACN showed the lowest ratio, but ANOVA did not indicate any significant difference (data not shown).

BSO treatment induces deep decline of tGSH and GSSG even at minimal doses. As expected, 24-h BSO treatment induced decrease of tGSH in each cell line. When using 1 μM BSO the entity of depletion was quite dissimilar in the various cell lines; the residual tGSH varied between 35 and 80%. Instead, after 10 μM BSO treatment, a profound depletion of tGSH was evident in each cell line and the residual tGSH was <15% in each cell line. Fifty and 100 μM BSO induced deeper decrease, with residual tGSH <4%, and reaching in some cases the detection limit (Fig. 2A).

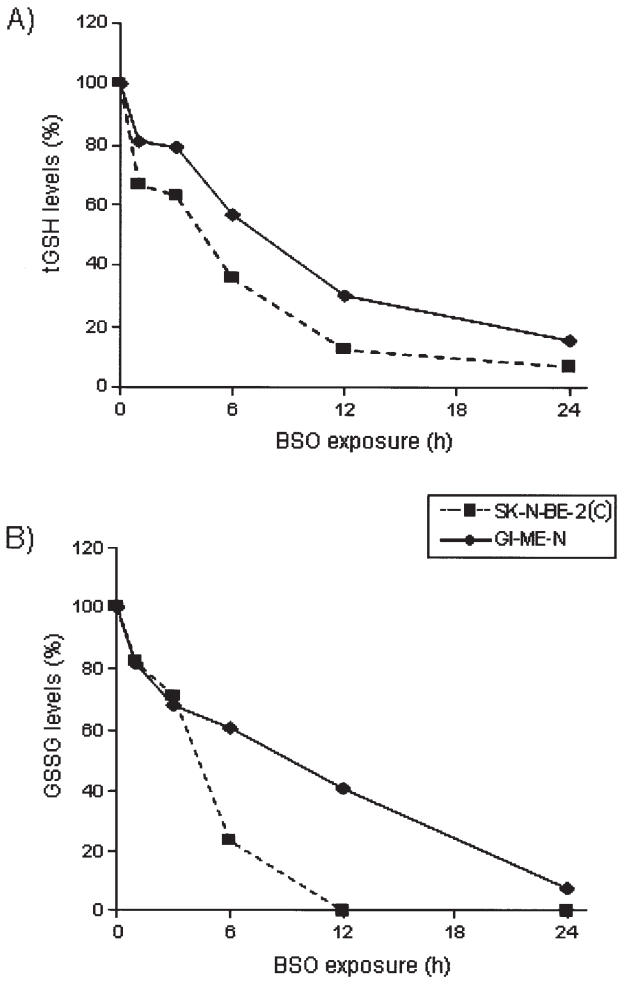

Figure 3. Changes in tGSH (A) and GSSG levels (B) in GI-ME-N and SK-N-BE-2(C) cells exposed to BSO (10 μM) for the indicated times. The concentrations of the analytes were determined by HPLC analysis and expressed as % of the control value.

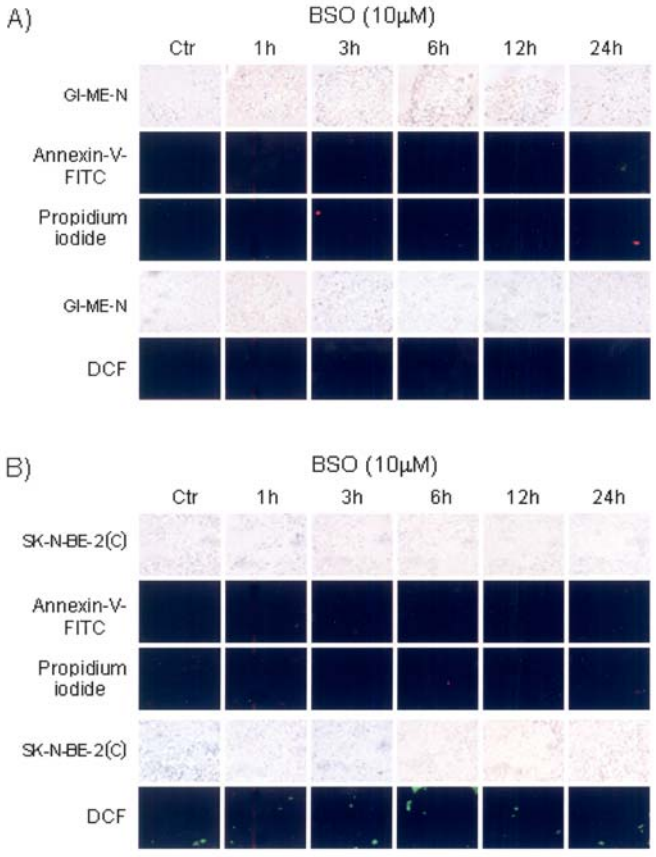

Figure 4. Apoptosis, necrosis and ROS generation in GI-ME-N (A) and SK-N-BE-2(C) (B) cells exposed to BSO (10 μM) for the indicated times. Apoptosis and necrosis were assessed by showing Annexin-V- and propidium iodide-positive cells. ROS generation was estimated by showing DCF-positive cells. In panels A and B the first and fourth line of sub panels show the cells observed by standard filters; the second line of sub panels are representative images obtained by fluorescence microscopy analysis of Annexin-V-FITC-positive cells; the third line of sub panels are representative images obtained by fluorescence microscopy analysis of propidium iodide positive cells; the fifth line of sub panels are representative images obtained by fluorescence microscopy analysis of DCF-positive cells.

BSO treatment caused a depletion of GSSG parallel to that seen for tGSH. Since basal levels of GSSG are much lower than basal levels of tGSH, after BSO treatment several GSSG levels were under the sensitivity of the method, this occurred at ≥10 μM BSO for the MYCN amplified cell lines [LAN5 and SK-N-BE-2(C)] and for the ACN line, and at ≥50 μM for the other two non-amplified MYCN cells (GI-ME-N and SH-SY-5Y) (Fig. 2B).

In consequence of the parallel decrease of tGSH and GSSG, the GSSG/tGSH, GSH ratio did not show any increase after BSO treatment, and remained stable <2%, at least until GSSG levels were detectable (data not shown).

Time course of GSH decline caused by 10 μM BSO in two NB cells characterized by a different MYCN status. The time course of the BSO-induced tGSH depletion was evaluated

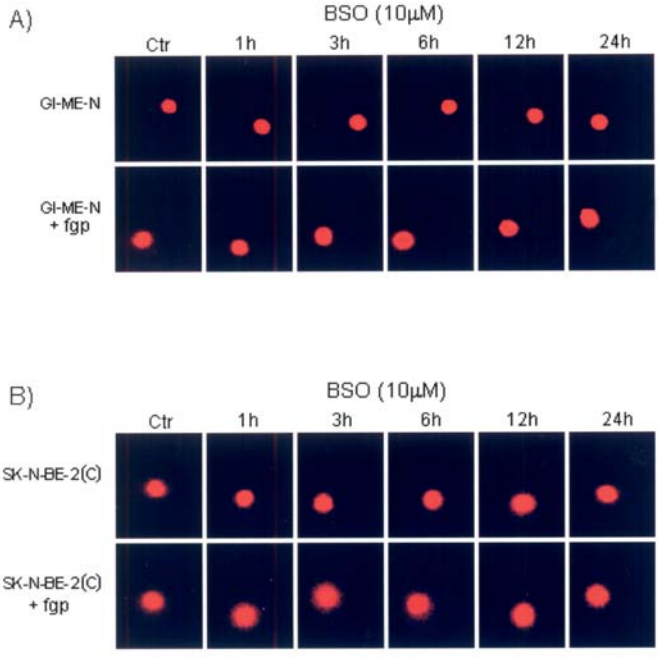

Figure 5. DNA damage in GI-ME-N (A) and SK-N-BE-2(C) (B) cells exposed to BSO (10 μM) for the indicated times. The lower sub panels in A and B show fgp-modified comet assay (oxidative DNA damage evaluation), the upper sub panels show standard comet assay.

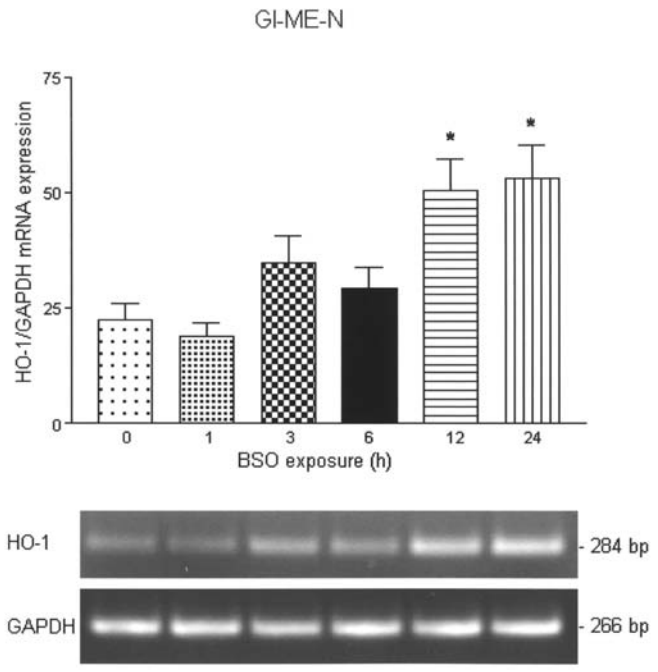

Figure 6. Real-time PCR analysis of HO-1 mRNA expression in GI-ME-N cell line treated with 10 μM BSO for the indicated times. The amount of target mRNA (obtained after densitometric analysis) was normalized to that of GAPDH mRNA from the same cDNA sample. Data are expressed as mean ± SEM. $^{*}p<0.05$ vs Ctr.

in two cell lines: GI-ME-N as a representative example of MYCN non-amplified cells, and SK-N-BE-2(C), as a representative example of MYCN amplified ones. In these experiments, BSO concentration was 10 μM, and the time course was monitored for 24 h. A progressive depletion of tGSH was observed for both cell lines; percent depletion was deeper in SK-N-BE-2(C) (Fig. 3A). A progressive decline was evidenced for GSSG as well; the decrease was more pronounced in SK-N-BE-2(C) cells, probably due to their low basal GSSG level, which went below the detection limit already at 12 h (Fig. 3B). However, during the time course experiments, the GSSG/tGSH ratio did not change significantly, remaining <2%, at least until GSSG levels were detectable (data not shown).

GSH and GSSG depletion induced by 10 µM BSO is not able to induce increase in ROS generation, alterations of cell viability or DNA damage. Intracellular ROS production and cell viability were analyzed in GI-ME-N and SK-N-BE-2(C) cells exposed for 1, 3, 6, 12 and 24 h to 10 μM BSO and we found no changes in Annexin-V or propidium iodide or DCF positive cells (Fig. 4). Comet assay also showed that exposure of these NB cells to a single dose of BSO (10 μM) for different times (1, 3, 6, 12 and 24 h) did not induce either oxidative or non-oxidative DNA damage (Fig. 5).

BSO (10 µM) causes a time-dependent induction of HO-1 in GI-ME-N cells. The HO-1 mRNA expression was increased in GI-ME-N cell line already at early times during 10 μM BSO incubation, but reached significance versus control only at 12 h (Fig. 6); the HO-1 expression in SK-N-BE-2(C) cell line was feeble, and no significant induction could be detected, at least in our experimental conditions (data not shown).

Discussion

Glutathione has pleiotropic effects promoting cell growth and broad resistance to therapy (32). Indeed, several studies have shown that high amount of GSH is associated with a chemoresistant phenotype in cancer cells (6) and that GSH depletion sensitizes many tumours to anticancer drugs (10,11,33,34).

In this work, we observed that MYCN amplified cell lines [LAN5 and SK-N-BE-2(C)] show lower amounts of tGSH and GSSG in comparison with non-amplified cells (GI-ME-N, ACN and SH-SY-5Y). Although the examined neuroblastoma cells have different basal levels of tGSH and GSSG, they undergo a comparable percent tGSH depletion after BSO treatment: after a 24-h exposure to increasing concentrations of BSO (1-100 μM) we have found a parallel decrease in tGSH and GSSG levels. GSH loss appears to be dependent on BSO concentration until 10 μM, which causes a profound depletion of tGSH in each cell line. Higher BSO concentrations deepened GSH depletion, but only slightly. A similar course has been registered for GSSG levels, and these findings are confirmed by GSSG/tGSH ratio, which do not undergo any significant variation in comparison to control conditions. Exposure of SK-N-BE-2(C) and of GI-ME-N to the critical dose of BSO (10 μM) for different hours (1, 3, 6, 12 and 24 h) induces tGSH and GSSG depletion, which is more dramatic in SK-N-BE-2(C). In BSO-treated GI-ME-N cells both tGSH and GSSG decrease is time-dependent.

Previously, Anderson and colleagues (15) have reported that MYCN-amplified cell lines are more sensitive to high doses of BSO (>1 mM) than MYCN-non-amplified cells and the degree of GSH depletion is variable but does not correlate with the degree of cytotoxicity measured as apoptosis rate. Analogously, our recent studies have demonstrated that a decrease of the intracellular tGSH pool achieved through administration of 1 mM BSO causes oxidative stress and apoptosis of SK-N-BE-2(C) neuroblastoma cells via activation of PKCδ (35,36).

In the present report, we show that 10 μM BSO is a sublethal dose that is not able to induce changes in ROS intracellular amount and it does not affect cell viability of either MYCN-amplified [SK-N-BE-2(C)] or non-amplified cells (GI-ME-N); moreover, the analysis of both standard and fgp-modified comet test is negative at all times of BSO incubation and in both cell lines thereby demonstrating that 10 μM BSO even if it drastically reduces intracellular tGSH levels, is not genotoxic. This significant reduction of intracellular redox state that does not influence cell viability might potentially enhance the sensitivity of neuroblastoma cells to subsequent chemical stress. Recently, it has been demonstrated that alteration of redox state in C6 glioma cells increases their susceptibility to cadmium toxicity (37).

Oxidants and agents able to modify cellular GSH levels (BSO, iodoacetamide) induce the expression of heme oxygenase-1 (HO-1) (38). HO-1, which is induced by a variety of stress stimuli and many cancer chemopreventive agents, represents a prime cellular defense mechanism against oxidative stress via antioxidant function of its

catabolic products, like bilirubin and carbon monoxide (39). The biological significance of HO-1 induction remains a matter of debate: reports have identified this induction as a prelude to cell death and others have described it as a mechanism of cell survival (40-44).

To investigate the reason why GSH depletion not accompanied by ROS generation does not affect neuroblastoma survival, we have analyzed HO-1 expression and we have found that it is increased in GI-ME-N cells, while its expression is not induced in SK-N-BE-2(C) cells. These findings demonstrate that GSH depletion *per se* is not sufficient to induce apoptosis of neuroblastoma cells but it is able to stimulate an increase in HO-1 gene expression at least in GI-ME-N cells, which show the highest basal levels of GSH. This protective response induced by BSO sublethal dose might explain the resistance to apoptosis of GI-ME-N cells exposed to higher concentrations of GSH depleting agent (1 mM BSO) (data not shown). Consistent with our results, it has been recently reported that neurons overexpressing HO-1 resist to oxidative stress-mediated cell death (42) and more recently that overexpression of HO-1 in human lung adenocarcinoma cells contributes to resistance to apoptosis induced by epigallocatechin 3-gallate (45).

Overall our study clearly demonstrates that, although deep GSH depletion can be obtained even with low BSO concentrations (10 μM), such a depletion alone is not sufficient to impair the survival of all the NB cells examined, and we suggest that modulation of HO-1 expression might be a potential therapeutic target against the survival of neuroblastoma cell lines more resistant to perturbations of oxidative status. However, in agreement with recent studies (37,46,47) we can speculate that the manipulation of redox state (e.g. by BSO) in neuroblastoma cells may be an initiating mechanism, helpful to reduce chemotherapy dosage and to overcome the risk of chemoresistance; the deep GSH depletion obtained with low BSO concentrations might suggest that low BSO doses could be sufficient; therefore, more in-depth studies will be necessary to better understand the effect of GSH depletion on drug sensitivity in neuroblastoma cells, and to define efficient doses and associations of GSH-depleting and antineoplastic agents.

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