Acta Derm Venereol (Stockh) 1989; 69: 137–141

Modulation of Glutathione Level in Cultured Human Melanoma Cells

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The effects of buthionine sulphoximine (BSO) treatment on cellular glutathione (GSH) content and on the cytotoxic action of menadione were investigated in cultured IGRI human melanoma cells. Addition of BSO (10^{-8}–0.5×10^{-3} M) to the cultures resulted in a dose- and time-dependent depletion of cellular GSH. BSO (10^{-5} and 10^{-6} M) did not influence cell multiplication up to 48 h, as determined by trypan blue staining. Menadione (3×10^{-5} M) treatment decreased the cellular GSH concentration and also reduced cell number after a 24 h exposure. Its cytotoxicity was increased by BSO (10^{-5}, 10^{-6} M), though the potentiating effect was moderate. ***Key words: Buthionine sulphoximine; Menadione; Cytotoxicity.***

(Accepted October 27, 1988.)

Acta Derm Venereol (Stockh) 1989; 69: 137–141.

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Glutathione is the major non-protein thiol of the cell and this tripeptide plays an important role in the reduction of the disulfide linkages of proteins, in synthesis of the deoxyribonucleotide precursors of DNA and in the cellular defence against oxidative stress and free radicals (1). Today we have a fairly comprehensive picture of glutathione (GSH) metabolism which facilitates the specific modulation of intracellular GSH levels.

Glutathione is synthesized in the cells from glutamic acid, cysteine and glycine, the reaction being catalysed by γ-glutamylcysteine synthetase and glutathione-synthetase. Intracellular GSH can be depleted among other drugs by buthionine–sulphoximine (BSO), a very effective and specific inhibitor of γ-glutamylcysteine synthetase, the rate limiting enzyme of the GSH synthesis (2). An increase in its synthesis and intracellular level can be achieved by increasing the substrates of the two synthetases.

The cytotoxic and antitumour activities of certain chemotherapeutic agents e.g. quinones, are thought to be related to an oxidative stress (3, 4). GSH may protect against these effects in several ways, including conjugation with the original compound or its semiquinone radicals, or by removing the formed hydrogen peroxide or hyperoxides produced by lipid peroxidation (5). Thus, an elevated GSH level may protect tumour cells, while depletion of intracellular GSH may potentiate the cytotoxic effect of these drugs.

In spite of the large volume of data on glutathione, the literature on its function in the melanocyte is sparse. Earlier studies suggested that GSH may play a role in human pigmentation. The question has been reviewed recently in a report where further information was provided on the importance of cysteine and GSH in melanin synthesis (6). Quite surprisingly a drastic change in intracellular GSH levels could be obtained without any significant interference with melanin precursors or melanoma cell proliferation.

In view of the above-mentioned data, it would seem reasonable to further explore the function of GSH in the melanoma cell with regard to melanin synthesis and tumour activity. The aim of the present study was to characterize the BSO-induced GSH level depletion in the melanoma cells and investigate the effect of BSO pretreatment on the cytotoxicity of the oxidative agent, menadione.

MATERIALS AND METHODS

Cell culture

Cultures of a pigment-producing human melanoma cell line (IGRI) were obtained from Dr Christian Aubert, Marseille, and since March, 1982 have been kept in culture at the Tornblad Institute, University of Lund, using methods described previously (7). We used Eagle's minimal essential medium (MEM) supplemented with 15% fetal calf serum (Flow Labs.). About 0.75×10^6 cells were seeded in 5 ml medium in polystyrene tissue culture flasks, volume 50 ml.

Analysis of cellular cysteine and glutathione

The total cysteine and glutathione content of the cells was determined after a prederivatization reduction with dithiothreitol (DTT, Merck) *ad modum* Cotgreave & Moldeus (8). After in situ derivatization of free and DTT-reduced thiols

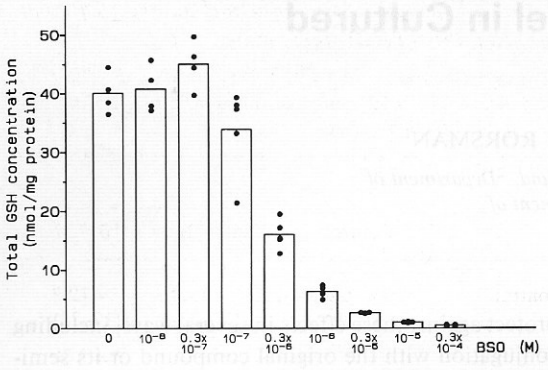

Fig. 1. Depletion of cellular GSH in IGRI melanoma cells by different BSO concentrations (values and means of separate experiments.)

with monobromobimane, HPLC was used to quantify strongly fluorescent bimane adducts. There were some minor modifications made in the liquid chromatographic step.

The separation was performed isocratically on a 150×4.6 mm stainless steel column packed with 3 µm Supelcosil LC-18 (Supelco, Bellafonte, Pa., USA). The use of a precolumn substantially prolonged the lifetime of the analytical column. The precolumn was a Waters Guard PAK™, resolve C_{18} (Waters, Milford, Mass., USA). Its insert was changed weekly. The isocratic conditions using an acetonitrile/acetic acid buffer at pH 3 were maintained for 7 min and a step gradient was used to wash out late-eluting compounds.

Effect of buthionine sulphoximine on melanoma cell GSH

After 2 days of culture, media were renewed and buthionine sulphoximine (Sigma) was added to melanoma cells in a final concentration of 10^{-8}, 0.3×10^{-7}, 10^{-7}, 0.3×10^{-6}, 10^{-6}, 0.3×10^{-5}, 10^{-5}, 0.3×10^{-4} M. Cells were harvested and analysed for thiols after 24 h of incubation with BSO. In the second part of the experiment, medium was replaced after 2 days of replating, with new medium containing 0.5×10^{-3} M BSO. Cells were exposed for 3, 6, 12 or 24 h to the drug before thiol determination.

Effect of menadione on melanoma cell culture

After incubation for 2 days the medium of the cultures was removed and replaced by fresh medium containing 3×10^{-5} or 10^{-5} M menadione sodium bisulfite (Sigma). (In a pilot study, a menadione concentration of 10^{-4} M was found to kill all of the cells as early as 4 h after application, whereas concentrations of 10^{-5} and 10^{-6} M had no effect on cell multiplication even after 48 h; unpublished observations.) GSH was assayed after a 24 h incubation with the drug.

Effect of menadione on buthionine–sulphoximine-pretreated melanoma cells

For assay of menadione effect on BSO-modulated melanoma cells, a small volume (10 µl) of buthionine–sulphoximine was added to the cultures without renewing the medium after 24 h of replating. The final concentration of BSO was 10^{-5} or 10^{-6} M, respectively. After incubation for 24 h the medium was removed and replaced with medium containing BSO (10^{-5}, 10^{-6} M) or BSO (10^{-5}, 10^{-6} M) and menadione in 3×10^{-5} or 10^{-5} M concentrations. Cells were harvested and analysed for thiols 24 h after renewal of media.

In all sets of experiments, controls were treated in the same way as test samples, except for the non-addition of the drugs to the medium.

Vitality

Parallel to the thiol assay the vitality of melanoma cells was also determined after incubation with 10^{-5} or 10^{-6} M BSO (48 h), 3×10^{-5} or 10^{-5} M menadione (24 h) and the combined application of the two drugs. Cells were harvested with 0.25% trypsin solution and counted with trypan blue added to distinguish living from dead cells. The numbers of living cells in test flasks were compared with those in the corresponding test or control flasks.

Protein

The assay was done according to the method of Lowry et al. (9).

RESULTS

Effect of BSO on melanoma cell GSH

BSO (0.3×10^{-4}–10^{-8} M) caused a dose-dependent depletion of GSH (Fig. 1). After 24 h exposure to 0.3×10^{-4} M a maximum depletion to 1.2% of control was obtained. The effect of the drug decreased gradually when the dose was reduced. At 0.3×10^{-7} and 10^{-8} M concentrations there were no more marked alterations in glutathione concentration.

The BSO (0.5×10^{-3} M) induced GSH depletions were found to be time dependent (Fig. 2). A depletion to about 50% of control was observed after 3 h of incubation. The GSH level decreased further with time and after 24 h exposure to the drug it reached its minimum at 0.99%.

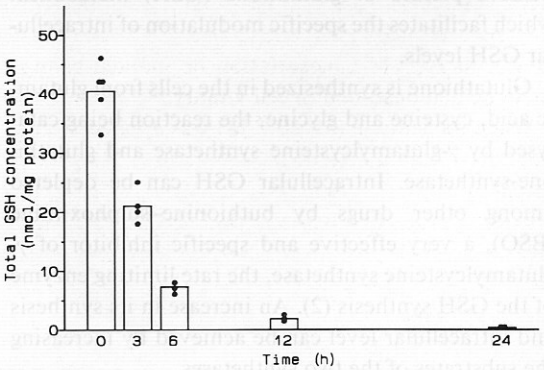

Fig. 2. Depletion of cellular GSH in IGRI melanoma cells exposed to 0.5×10^{-3} M BSO for different time intervals (values and means of separate experiments.)

Table I. *Effect of BSO and/or menadione treatment on cellular GSH content of IGRI melanoma cells*

The data are expressed as nmol GSH/mg protein (mean and range of 6 samples). ND = not detectable

		BSO	
	Control	10^{-5} M	10^{-6} M
Control	$\bar{x}$=32.7 (28–39)	$\bar{x}$=1.1 (0.7–2.0)	$\bar{x}$=7.0 (5.2–8.6)
Menadione 3×10^{-5} M	$\bar{x}$=19.7 (15–28)	$\bar{x}$=0.2 (ND–0.6)	$\bar{x}$=1.8 (0.4–4.4)
10^{-5} M	$\bar{x}$=32.5 (24–38)	$\bar{x}$=0.6 (0.5–0.7)	$\bar{x}$=5.4 (5.0–5.8)

Effect of menadione on melanoma cell culture

Menadione in a 3×10^{-5} M concentration reduced the intracellular GSH concentration considerably, though with 10^{-5} M it had no effect (Table I).

Effect of BSO and menadione treatment on melanoma cell GSH

The low intracellular GSH concentration induced by BSO (10^{-5} or 10^{-6} M) was further reduced by both concentrations of menadione (3×10^{-5}, 10^{-5} M). The effect was small though distinct, compared with BSO treatment (Table I).

Effect of BSO and/or menadione on cell vitality

The proportion of trypan blue stained cells varied between 5% and 8%, in both control and test flasks.

BSO (10^{-5} or 10^{-6} M) did not influence cell multiplication after 48 h of incubation, as determined by trypan blue staining (Table II).

Menadione in 3×10^{-5} M concentration considerably reduced the cell number after 24 h exposure, though in 10^{-5} M concentration no marked change could be demonstrated in cell vitality.

When menadione (3×10^{-5} M) was applied after a 24 h pretreatment with BSO (10^{-5} or 10^{-6} M), a small potentiation of cell toxicity was observed. This effect was almost the same, regardless of its concentration. The cytotoxic effect of the lower menadione dose (10^{-5} M) was not increased by either of the BSO concentrations.

DISCUSSION

Buthionine sulphoximine has been shown to decrease GSH concentration in various cells grown in tissue culture (10). The drug has also been used in studies on melanoma cell cultures to investigate the effect of thiol depletion on the concentration of melanin precursors (6), and recently a report was published concerning the effects of BSO on the cytotoxicity and DNA cross-linking induced by bifunctional DNA-reactive agents in a human melanoma cell line (11).

Table II. *Effect of BSO and/or menadione treatment on the vitality of IGRI melanoma cells*

Determinations were made on 3rd day of culture. The data are expressed as $\times10^5$ cell/flask (mean and range of 5 samples). ND = not detectable

		BSO	
	Control	10^{-5} M	10^{-6} M
Control	$\bar{x}$=16.8 (9.4)[a] (14–19)	$\bar{x}$=19.0 (16–23)	$\bar{x}$=19.2 (17–23)
Menadione 3×10^{-5} M	$\bar{x}$=3.1 (ND–8.0)	$\bar{x}$=0.08 (ND–0.2)	$\bar{x}$=0.06 (ND–0.1)
10^{-5} M	$\bar{x}$=15.8 (14–18)	$\bar{x}$=13.6 (8–16)	$\bar{x}$=15.0 (11–20)

[a] Numbers in parentheses, $\times10^{-5}$ cell/flask on 2nd day of culture (mean of 5 samples).

The present results demonstrate that BSO causes a dose- and time-dependent depletion of the cellular GSH content in melanoma cell culture, thus providing a suitable model for the study of the effect of cellular thiol deprivation.

The greater capacity of BSO to reduce cellular GSH in our experiments, compared with that of the above-mentioned study with bifunctional chemotherapeutic agents (11), may be attributed to the use of different melanoma cell lines (IGRI versus RPMI 8322) and/or to differences in culture methods.

According to our results, cell multiplication was not inhibited by BSO (10^{-5} and 10^{-6} M) even after 48 h and in spite of the low glutathione concentration. Several reports have demonstrated that a reduction of GSH concentration does not influence the rate of cell growth, the amount of cell protein, or the chromosome structure during culture for at least 24 h (11–13). However, for longer exposure times, toxicity and growth inhibition were demonstrated in a dose-dependent fashion (14–16). Doses of 10^{-7} and 10^{-5} M BSO did not inhibit growth even following up to 75 h of exposure, whereas even 5×10^{-5} M caused a slight decrease in the final cell density achieved. Exposure to 10^{-4} M resulted in a significant cell loss at

exposure times beyond 50 h in cultured EMT6/SF mouse tumour cells (16). In rat heart cell cultures, BSO did not influence the multiplication rate over a 5-day period even at a dose of 10^{-4} M (15), though in murine mammary carcinoma cells, 0.5×10^{-4} M was found to be toxic already after 48 h (14). These data indicate that BSO toxicity may well be cell-type dependent as well, though doses of 10^{-5} M and less seem to be well tolerated in different cell types.

Menadione has been extensively studied with regard to quinone toxicity and its reactions with GSH (4). Its toxicity is mediated via 1-electron reduction (4, 17), which may lead to DNA strand breaks and damage of cell membrane and other vital cellular constituents (18, 19).

Menadione was reported to cause a rapid, concentration-related depletion of GSH in isolated rat hepatocytes (20). However, in a human colonic tumour cell line, menadione resulted in pronounced (but statistically non-significant) depletion, due to the large variation in the response (12). Our results confirm the data on hepatocytes with a dose-dependent decrease in melanoma cell GSH after menadione treatment. Menadione also further reduced the BSO-depleted GSH content of the cells.

In the present experiments, BSO moderately potentiated menadione cytotoxicity. However, it was of interest that though inducing considerably different glutathione depletion, both BSO concentrations resulted in a similar potentiation of toxicity.

Similar results—namely small but significant potentiation of menadione cytotoxicity by BSO pretreatment of different cell types—have been reported by other authors as well (12). GSH may protect against quinone-mediated oxidative stress in several ways, including removal with glutathione peroxidase either H_2O_2 formed or hydroperoxides produced as a result of lipid peroxidation (5). The oxidized glutathione formed by this reaction is reduced by NADPH-dependent glutathione reductase (20). In isolated rat hepatocytes, NADPH oxidation occurs rapidly after menadione exposition (4) and a correlation was observed between the failure of NADPH recovery and lethal cell injury (20). However, it has been demonstrated recently that inhibition of glutathione reductase does not prevent the rapid oxidation of NADPH, suggesting that glutathione reductase is not the primary source of NADPH consumption during menadione-induced oxidative cell injury (20).

In the case of the nitrogen analogues of the quinones, the quinone(di)imines, it has been demonstrated that their toxicity is not correlated with the rate of their addition to reduced glutathione (21). Though the difference in chemical structure might be of deciding importance, this latter observation probably also indicates the potential significance of other NADPH-consuming pathways and/or cytotoxic mechanisms of quinones. This possibility could also explain the modest and (according to our results) dose-independent potentiating effect of glutathione depletion on menadione toxicity.

Since BSO pretreatment does not seem to be a very effective way to enhance menadione cytotoxicity in cultured cells, the potential chemotherapeutic use of the combination is doubtful.

However, as BSO was found to reduce cellular glutathione to a few per cent of the normal value without influencing cellular vitality, for at least 2 days, it would seem worthwhile to look for new combinations, i.e. for drugs, whose toxicity would be specifically and more significantly increased by thiol depletion. On the other hand, further investigation of the mechanism of effects of quinones may allow a more specific and thus more effective interference with their metabolism and toxicity, too.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Cancer Society (project 626-B89-17XB), the Swedish Medical Research Council, the Walter, Ellen and Lennart Hesselman Foundation for Scientific Research, the Edward Welander Foundation for Scientific Research, the Thure Carlsson Foundation for Scientific Research, the Crafoord Foundation for Scientific Research, the donation funds of the University Hospital at Lund, and the donation funds of the Faculty of Medicine, University of Lund.

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