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Report

Role of γ-glutamyltranspeptidase on the response of poorly and moderately differentiated rhabdomyosarcoma cell lines to buthionine sulfoximine-induced inhibition of glutathione synthesis

Begoña Castro,[1] Ana Alonso-Varona,[1] Maite del Olmo,[1] Pedro Bilbao[2] and Teodoro Palomares[2]

[1]Department of Cell Biology and Morphological Sciences, and [2]Department of Surgery, Radiology and Physical Medicine, School of Medicine and Dentistry, University of the Basque Country, Leioa, 48940 Vizcaya, Spain.

Glutathione (GSH) is involved in many cellular functions, including cell growth and differentiation. GSH also plays an important role in the protection of cells against oxidative damage and hence in determining the sensitivity of cells to the cytotoxicity of anticancer agents. Because of this, induction of GSH depletion has been proposed as a good strategy for sensitizing tumor cells to antitumor agents. The aim of the present work is to study the effect of buthionine sulfoximine (BSO, a specific cellular GSH-depleting agent) in two rat tumor cell lines derived from the same rhabdomyosarcoma tumor model, the moderately differentiated and low metastatic F21 cell line, and the poorly differentiated and high metastatic S4MH cell line, to investigate the influence of the degree of differentiation in the induction of GSH depletion-based therapy. We observed that, whereas in the S4MH cell line BSO induced a dose-dependent inhibition of both cell growth *in vitro* and tumorigenic potential *in vivo*, in F21 cells the administration of moderate doses of BSO enhanced tumor growth and only at high doses was there a slight reduction of their tumorigenic potential. These effects were in consonance with the fact that the activity of γ-glutamyltranspeptidase (γ-GT) present in the F21 cells was 4 times higher than in the S4MH cells. Indeed, inhibition of γ-GT activity by acivicin not only abrogated the BSO-induced increase of GSH content and of cell growth, but also the combination of acivicin + BSO significantly decreased intracellular GSH levels and cell proliferation, and induced F21 cells to apoptosis. These studies suggest that, as occurs in the rhabdomyosarcoma tumor model, γ-GT levels and the degree of differentiation of tumor cells might influence the response of tumor cells to inducers of GSH depletion, and should be taken into account in therapies based on GSH metabolism. [© 2002 Lippincott Williams & Wilkins.]

Key words: Buthionine sulfoximine, differentiation, γ-glutamyltranspeptidase, glutathione, proliferation.

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Correspondence to T Palomares, Departamento de Cirugía, Radiología y Medicina Física, Facultad de Medicina y Odontología, Universidad del País Vasco, Leioa, 48940 Vizcaya, Spain.
Tel: (+34) 94 601 2813; Fax: (+34) 94 464 92 66;
E-mail: ocppacat@lg.ehu.es

Introduction

The oxidative modification of cell components via reactive oxygen species (oxidative stress) is one of the most potentially damaging processes for proper cell functions.[1] Glutathione (GSH; L-γ-glutamyl-L-cysteinylglycine), the major intracellular non-protein thiol, plays an important role in protection against oxidative damage caused by reactive oxygen species that may be formed normally in metabolism and from some exogenous sources.[2] GSH is also involved in other normal cellular activities, including protein synthesis, cell growth and differentiation.[3,4] It has been proved that GSH synthesis is essential for normal growth[5] and that as the cell progress from proliferation to differentiation, cellular GSH content is decreased.[3] This led to the notion that thiol status is dependent on cellular energy metabolism.

With respect to tumor cells, it has been shown that, for example, non-differentiated high metastatic melanoma cells have a significantly higher GSH content than non-tumorigenic melanocytes.[6] Moreover, it has been demonstrated that whereas elevation of intracellular GSH is associated with mitogenic stimulation,[7] GSH depletion decreases the rate of cell proliferation and inhibits cancer growth.[8,9]

The fact that some tumor cells contain very high levels of GSH has led to the suggestion that it is an important factor in limiting the therapeutic efficiency of conventional cancer treatment. The most important function of GSH in chemotherapy is that it is involved in the detoxification of xenobiotics by the conjugation of the electrophilic group of the toxin to the nucleophilic sulfhydryl of GSH and consequently it protects important cellular nucleophilic macromolecules (e.g. DNA), determining the sensitivity of cells to drug-induced cytotoxicity.[10] Depletion of intracellular GSH by L-*S*,*R*-buthionine sulfoximine (BSO), a potent inhibitor of γ-glutamylcysteine synthetase (γ-GCS, the rate-limiting enzyme in GSH synthesis),[2] has a growth-inhibitory effect on several tumor cell lines and significantly enhances the cytotoxicity of many cytotoxic agents, as has been demonstrated in preclinical[11–13] and clinical studies.[14] It has been suggested that measurement of intracellular GSH content in tumors might allow selection of patients whose tumors contain lower GSH levels for alkylating agent-based chemotherapy and those whose tumors have higher GSH levels for strategies aimed at lowering tumor GSH content. However, since GSH is a complex and dynamic process that concerns the activity of several related enzymes, it may be difficult to know *a priori* what type of GSH metabolism a tumor may have and, in particular, what influence its degree of differentiation has in the response to a BSO-based therapy for the GSH metabolism. Because this could be important with a view to using this GSH modulator as a chemosensitizing agent, the present study investigates the possible differences in GSH levels and tumor growth between the moderately differentiated and low metastatic F21 cell line, and the poorly differentiated and high metastatic S4MH cell line, derived from the same rat rhabdomyosarcoma tumor model, when they are exposed to BSO-induced inhibition of GSH synthesis.

Materials and methods

Tumor cell lines

Two variants from the same parental rhabdomyosarcoma cell line with different metastatic potential were used: S4MH (highly metastatic) and F21 (poorly metastatic).[15] Cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 15 and 10% fetal calf serum (FCS), respectively, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma, St Louis, MO) in a humidified atmosphere (5% CO_2, 95% air) at 37°C.

Exponentially growing cell cultures were used in all experiments. After a brief exposure to phosphate-buffered saline (PBS)/EDTA (2 mM) and centrifuging, the pellet was re-suspended in complete medium plus FCS and cells were enumerated with a Coulter counter (Coultronics, Margency, France). Viability, as determined by Trypan blue exclusion, ranged from 95 to 98%.

Drugs

BSO (an inhibitor of γ-GCS) and acivicin [a non-competitive inhibitor of γ-glutamyltranspeptidase (γ-GT)] were obtained from Sigma (St Louis, MO). For the *in vitro* studies the drugs were dissolved in DMEM. For the *in vivo* experiments BSO was dissolved in sterile physiological saline solution (0.9% NaCl) adjusted to pH 7.2 and administered i.p. Dosages used were adjusted for body weight.

Cell proliferation

Tumor cells were seeded in 24-well microplates at a density of 10^4 cells/well in 10^3 μl of growth medium plus FCS and allowed to attach and grow for 24 h. The cells were then treated with BSO, acivicin or acivicin plus BSO. After treatment, cells were washed free of drugs and allowed to grow for an additional 72 h. At 24, 48 and 72 h after addition of drugs, proliferation was measured by using a hemocytometer to count the cells growing in each well. Experiments were performed in quadruplicate and each assay was repeated 3 times.

GSH determination

Cells were harvested by a brief exposure to PBS/EDTA (2 mM), centrifuged and resuspended at a concentration of 10^6 cells/ml in DMEM. In order to determine the intracellular GSH content, cell samples were incubated in the presence of monochlorobimane (Molecular Probes, Eugene, OR) at a concentration of 20 μM in PBS for 10 min at room temperature in the dark. The fluorescence emitted was measured by flow cytometry. At least five samples of 10^4 cells were analyzed on an Epics Elite flow cytometer (Coulter, Hialeah, FL), as described elsewhere.[5] Briefly, the stained cells were excited at 360 nm and both forward and 90° light scatter were measured using a 460 nm long pass filter. Non-viable

cells in each sample were detected by the addition of propidium iodide (0.5 μg/ml) to each sample immediately before flow cytometry analysis. Strongly propidium iodide-stained cells corresponded to dead cells and were excluded for data acquisition purposes.

Detection of apoptosis

Apoptosis was detected by analyzing the reduced fluorescence of DNA-binding dye propidium iodide in the apoptotic nuclei due to DNA fragmentation.[16] Cells were harvested and fixed in 70% ethanol at −20°C for 30 min. Samples were washed twice (5 min, 450 *g*) in PBS and resuspended at a concentration of 10^6 cells/ml in a PBS solution containing propidium iodide (50 μg/ml) and RNase (76 U/ml) for 30 min at room temperature in the dark. Fluorescence emitted per cell unit was measured by flow cytometry using an Argon laser (λ_{ex}=488 nm; λ_{em}=620 nm). Fluorescent signals emitted were collected with appropriate filters for propidium iodide (DL 488 nm, DL 550 nm, BP 525 nm, DL 600 nm, BP 675 nm). All the experiments were repeated 3 times.

Measurement of γ-GT

Activity of γ-GT was assayed essentially according to Grisk *et al*.[17] using γ-glutamyl-*p*-nitroanilide (Sigma) as substrate and glycyl-glycine (Sigma) as γ-glutamyl moiety acceptor. In a typical experiment, 10^6 cells were incubated for 30 min at 37°C in 0.75 ml of substrate with or without 40 mM glycyl-glycine in 5 mM Tris–HCl buffer, pH 8.5. The production of free *p*-nitroanilide was measured by spectrophotometry at 405 nm. Experimental values were obtained by subtracting the value of samples without glycyl-glycine from the value of samples with glycyl-glycine. The units of enzyme activity were calculated using a molar extinction coefficient of 9.9 for *p*-nitroanilide formed, where 1 U of γ-GT is defined as 1 μmol/min of substrate transformed/ml/min. Enzyme activity was expressed as mU of γ-GT activity/mg cell protein. Protein content was determined by the method of Lowry.[18]

Animals and tumor model

Specific pathogen-free female Wistar (WAG) rats (Iffa Credo Laboratories, L'Arbreole, France) were used for *in vivo* studies. Rats (10–12 week old) were given food and water *ad libitum*, and kept on a 12 h day/night cycle. The animals were handled according to institutional ethical guidelines and all protocols complied with the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia.[19]

Each experimental group consisted of eight rats, which were inoculated s.c. into the back with 0.1 ml of cell suspensions of syngeneic F12 or S4MH tumor cells at a concentration of 10^7 cells/ml in HBSS.

Measurement of tumors and drug activity evaluation

One set of experiments was focused on evaluating the effect on tumorigenic potential of F21 and S4MH cells previously exposed *in vitro* to BSO. The tumors were evaluated 3 times per week by the same observer, and the tumor weight (g) was determined by measurements of the longest axis (L) and shortest axis (W) of each tumor with a slide caliper and calculated assuming unit density by the following formula $(L \times W^2)/2$. The effect of BSO on tumorigenicity of F21 and S4MH cell lines was evaluated until day 26 after tumor cell inoculation as the ratio of the mean of weights of tumors in rats that were inoculated with BSO-treated cells to that in the control rats (T/C ratio). In addition, we investigated the effects of *in vivo* BSO administration on tumor growth in rats bearing F21 or S4MH tumors. For this set of experiments, BSO treatment was initiated when the tumor weight reached 50–150 mg (day 0) and was administered daily for 14 days. Tumor volumes were estimated by the formula $(L \times W^2)/2$. Relative tumor volume (RTV) was calculated using the formula $V_X/V_i \times 100$, where V_X represents the tumor volume on a given day of measurement and V_i is the volume of the same tumor at the start of treatment. BSO effect on tumors was evaluated using two different methods: by calculation of the T/C values, i.e. by dividing the RTV of treated rats by that of control rats, and by the growth delay factor (GDF), which was defined as the mean number of tumor-doubling times gained by treatment calculated with the formula: $GDF=(TD_{tr}-TD_{con})/TD_{con}$. In this formula, TD_{tr} is the tumor-doubling time of treated tumors and TD_{con} that of untreated tumors. Homogeneity in the distribution of the different experimental groups with respect to tumor volume before the beginning of the treatment was verified by statistical analysis (analysis of variance with one factor). On the day following treatment all rats were killed by cervical dislocation.

Statistical analysis

The test of significance was carried out using Student's *t*-test, the χ^2-test and the factorial analysis

of variance (ANOVA), as appropriate. The values were considered to be statistically different from controls when $p < 0.05$.

Results

Time course of GSH levels, and cell proliferation of S4MH and F21 cell lines

Initially we determined *in vitro*, under basal conditions, the differences between S4MH and F21 rhabdomyosarcoma cell lines on the time course of GSH levels and cell proliferation. A good positive correlation (r=0.78, $p < 0.001$) was found between these parameters. No significant differences in the GSH levels of the two tumor cell lines were found in the first 6 h of culture. However, at 24, 48, and 72 h a significant increase in GSH content (157, 132 and 183%, respectively) was observed in S4MH cells with respect to F21 cells (Figure 1A). Also, a significant difference in cell proliferation between these tumor cell lines was found (Figure 1B). Thus, at 72 h the proliferation of S4MH cells was twice that of F21 cells.

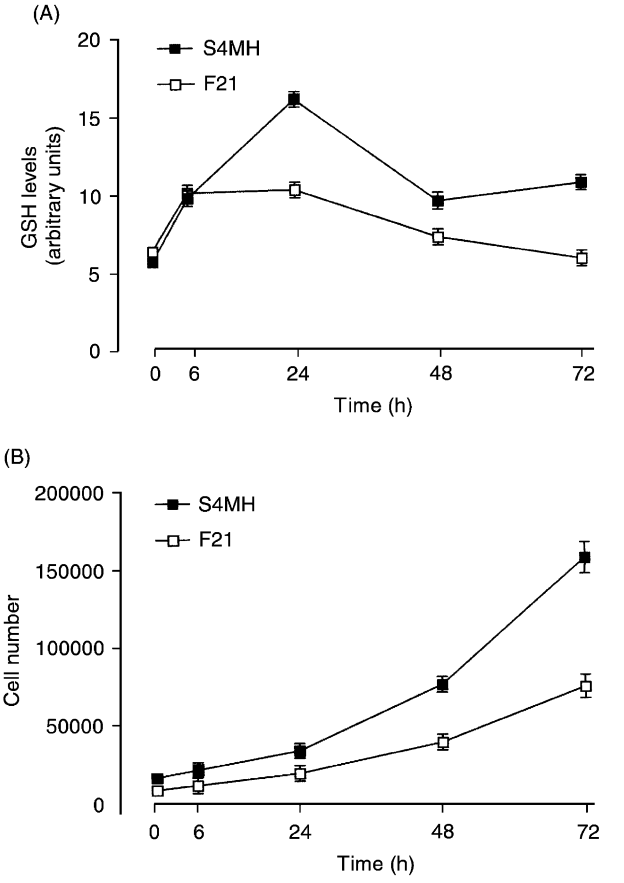

Figure 1. Time course of GSH levels (A) and cell proliferation (B) of S4MH and F21 cell lines under basal conditions. In order to determine the intracellular GSH content, cell samples were incubated in the presence of the fluorochrome monochlorobimane and the intensity of the fluorescence emitted was measured by flow cytometry. Cell proliferation was evaluated using a hemocytometer to count the cells growing in 24-well tissue culture plates. Experiments were performed in quadruplicate and the results are expressed as the mean $\pm$ SD of three experiments. Statistical differences were computed by Student's *t*-test ($p < 0.05$).

Effects of BSO on GSH content and proliferation of S4MH and F21 cells

BSO treatment significantly decreased the GSH content of S4MH in a concentration-dependent manner. Thus, after exposure to 100 and 500 μM of BSO (for 6 h), GSH levels were maximally reduced (34 and 66%, respectively) at 48 h (Figure 2A). Only with a dose of 100 μM BSO was any substantial recovery (up to 81% of control) observed in GSH levels at 72 h. In the case of the F21 cell line, a 39% reduction of intracellular GSH levels was observed with the dose of 500 μM BSO at 48 h, followed by a recovery up to the GSH levels of the control at 72 h. However, with the dose of 100 μM BSO no significant differences were observed with respect to untreated cells at 48 h, but a 33% increase of GSH content ($p < 0.05$) was observed in treated-F21 cells with respect to control cells at 72 h (Figure 2C).

Similarly, BSO-induced depression of GSH content was accompanied by a significantly concentration-dependent reduction of cell proliferation in the S4MH cell line (3.5 and 17.3 times lower with doses of 100 and 500 μM, respectively) with respect to untreated cells at 72 h (Figure 2B). The exposure of the F21 cell line to 500 μM BSO initially reduced cell proliferation compared to control cells at 24 and 48 h (1.8- and 1.4-fold, respectively, $p < 0.05$), but cell numbers were 87% of the control at 72 h. Exposure to 100 μM of BSO had no effect on the proliferation of F21 cells compared to untreated cells at 24 and 48 h. However, at 72 h cell proliferation was observed to be 1.5 times higher ($p < 0.05$) than for the control cells (Figure 2D).

Effects of BSO on γ-GT activity of S4MH and F21 cells

The differences observed between S4MH and F21 cell lines in terms of GSH levels and proliferation after treatment with BSO prompted us to investigated the possible involvement of γ-GT in this different response. Under basal conditions, both

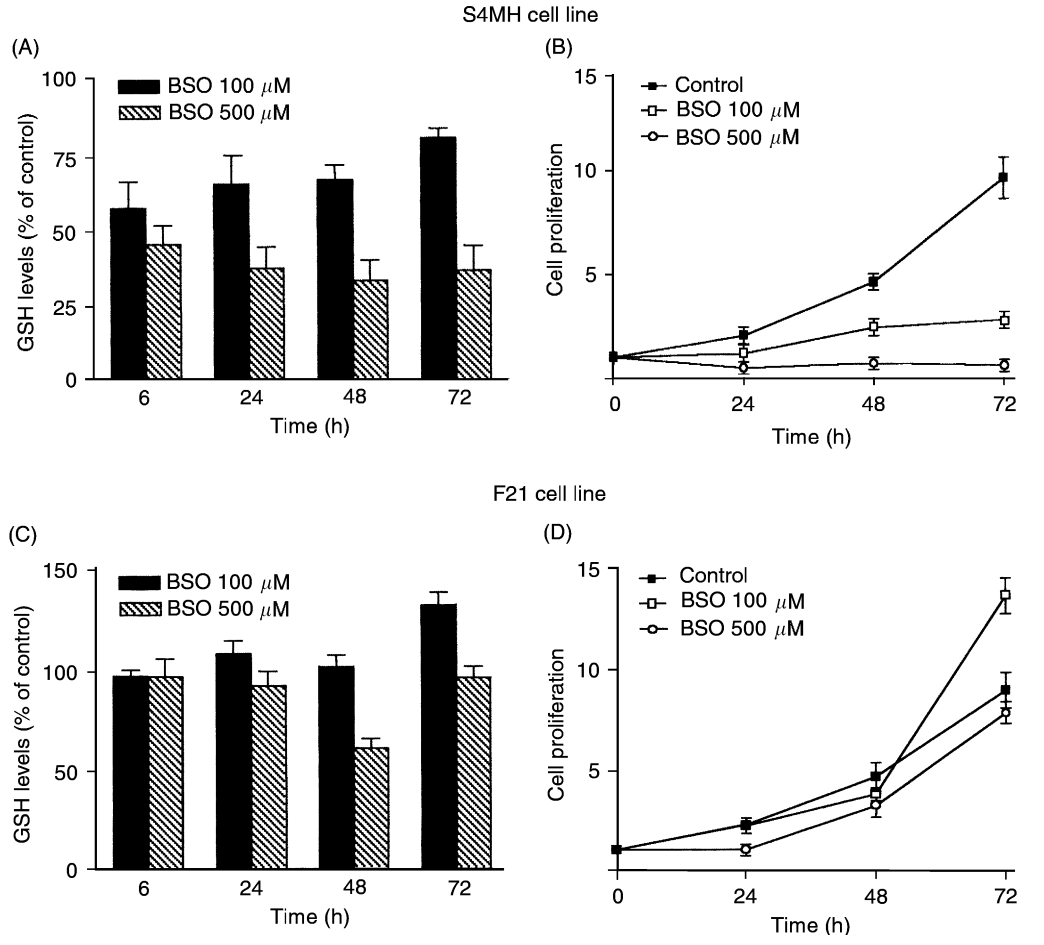

Figure 2. Effect of BSO (100 and 500 μM) on the GSH levels (A and C) and on cell proliferation (B and D) of S4MH and F21 tumor cell lines. GSH levels are expressed as a percentage of control values (i.e. for S4MH, BSO 100 μM, at 6 h, the GSH level was 55% of control value). Cell proliferation is expressed as relative values (mean $\pm$ SD) with respect to mean values of the control at 0 h. Experiments were performed in quadruplicate and each assay was repeated 3 times.

tumor cell lines showed measurable enzymatic activity, but the level of γ-GT activity was found to be 4 times higher in the F21 cells than in the S4MH cells (28.6 ± 1.5 versus 7.2 ± 0.6 mU/mg protein, $P < 0.05$). When tumor cells were exposed to 100 or 500 μM of BSO, a significant decrease in γ-GT activity took place in S4MH cells (4.4 ± 0.4 and 2.4 ± 0.2 mU/mg protein, respectively). In contrast, in F21 cells γ-GT activity was significantly increased (1.4 times that of control cells) by BSO treatment, with no significant differences between doses (41.0 ± 0.9 versus 38.6 ± 0.9 mU/mg protein; Figure 3).

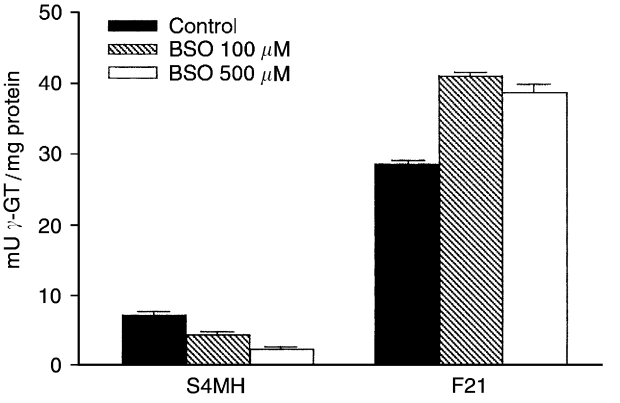

Figure 3. γ-GT activity of S4MH and F21 cells under basal conditions (Control) and after exposure to 100 and 500 μM of BSO. Enzyme activity is expressed as mU/mg of cell protein. Protein content was determined by Lowry's method. Data represent the mean $\pm$ SD of three experiments.

Effects of γ-GT inhibition on the response of S4MH and F21 cells to BSO treatment

Based on the differences observed in γ-GT between the two cell lines, we next investigated the effect of

γ-GT inhibition by acivicin on the response of S4MH and F21 cell lines to BSO treatment. We first determined that γ-GT activity was reduced by acivicin in a dose-dependent manner (data not shown). Exposure to acivicin (for 10 h) also produced a dose-dependent reduction of GSH levels (Figure 4A) and cell growth (Figure 4B), and an increase in the percentage of apoptotic cells (Figure 4C) in both cell lines. However, this effect was much more pronounced in F21 cells than in S4MH cells. Since significant differences between these cell lines were observed even with the lower dosage of acivicin, most subsequent experiments were performed only with this concentration (1 μM). As can be seen in Figures 5 and 6, the exposure of F21 cells to 1 μM of acivicin decreased GSH levels by 30% and resulted in a 1.3 times lower growth rate and a 3.2 times higher percentage of apoptotic cells as compared to untreated cells at 48 h of culture, whereas in the case of S4MH no significant modification was observed in these measured parameters with respect to control cells. In contrast, exposure to 100 μM BSO produced a significant increase (18.3-fold) of apoptotic cells in the S4MH treated cells compared to control ($p < 0.01$), but no significant differences were found in F21 tumor cells. Moreover, when BSO (100 μM) was added to S4MH cells previously exposed to acivicin no significant differences were observed in GSH levels, cell growth or the percentage of apoptotic cells with respect to cells treated with BSO alone. However, in the case of the F21 cell line not only did acivicin treatment abrogate the BSO-induced increase in GSH content and in cell growth observed in these tumor cells at 72 h of culture, but also the combination of acivicin+BSO decreased intracellular GSH levels by 38%, halved cell proliferation and increased the percentage of apoptotic cells to 3.4 times that of untreated cells ($p < 0.01$).

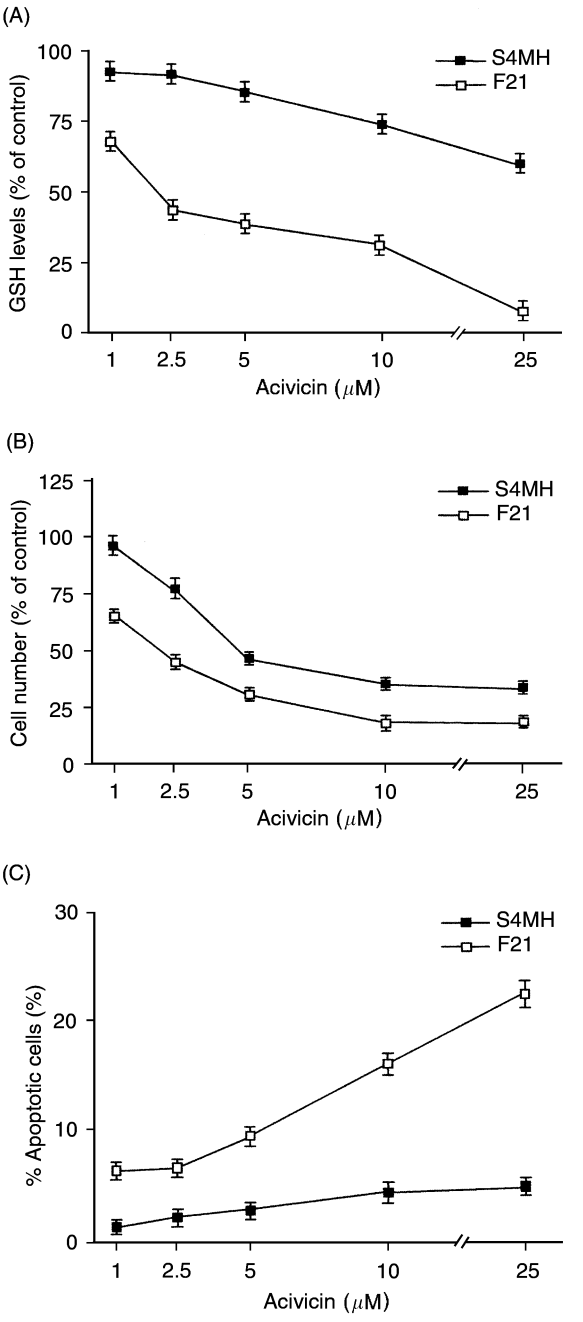

Figure 4. Effect of γ-GT inhibition by acivicin on the response of S4MH and F21 cells to BSO treatment. (A) GSH levels expressed as percentage of controls, (B) cell number expressed as percentage of untreated cells, and (C) percentage of apoptotic cells on S4MH and F21 acivicin-treated cultures. Data represent the mean $\pm$ SD of three experiments.

Effects of *in vitro* BSO pretreatment on tumorigenic potential of S4MH and F21 cells

Firstly, we compare the tumorigenic potential between the two tumor cell lines under basal conditions. We observed that on day 26 after tumor cell inoculation S4MH tumor weight was 6 times higher ($p < 0.01$) than F21 tumor weight (4.5 ± 0.9 and 0.7 ± 0.1 g, respectively).

Secondly, we evaluated the effect of BSO on the tumorigenic potential of S4MH and F21 cells. These tumor cells were cultured in the presence of 100 or 500 μM of BSO for 6 h and injected into rats 18 h after BSO was removed from the culture medium (when major BSO-induced GSH differences between the two cell lines were observed as described above). As shown in Figure 7, BSO treatment significantly

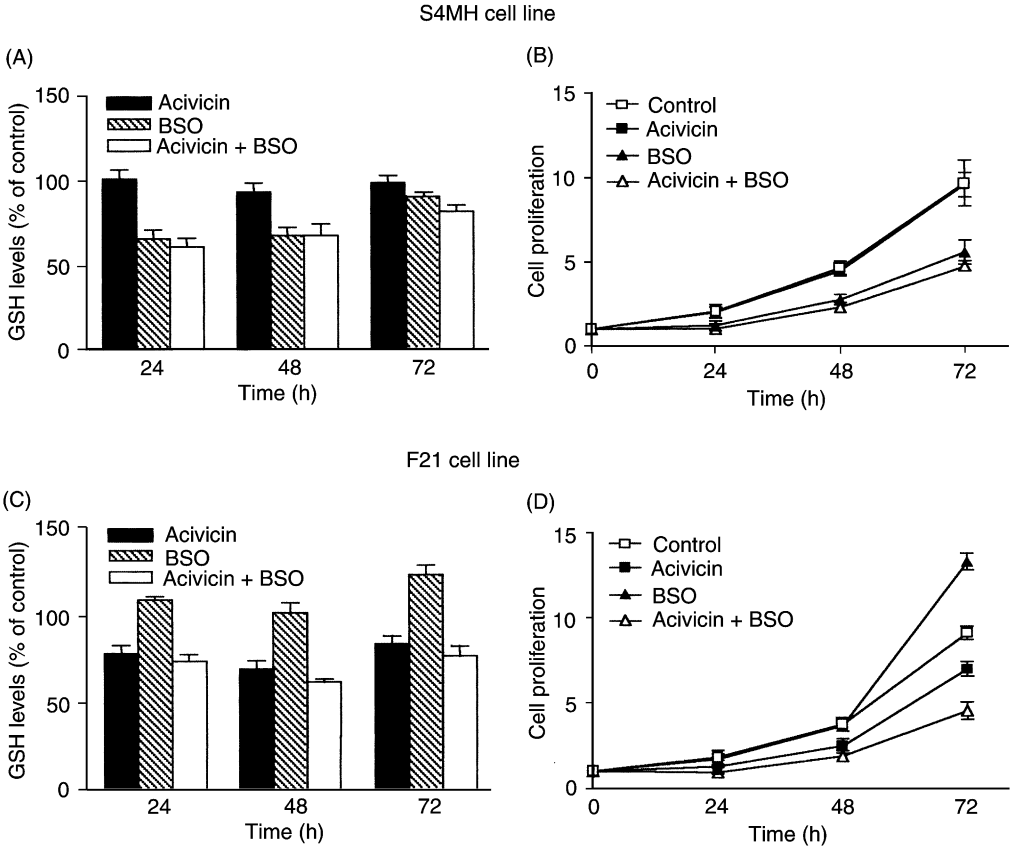

Figure 5. Effect of BSO treatment (100 μM) on intracellular GSH levels (A and C) and on cell proliferation (B and D) of S4MH and F21 cells cultured in control medium or previously exposed to acivicin (1 μM) for 10 h. GSH levels are expressed as a percentage of control values. Cell proliferation is expressed as relative values (mean $\pm$ SD) with respect to mean values of the control at 0 h. Experiments were performed in quadruplicate and each assay was repeated 3 times.

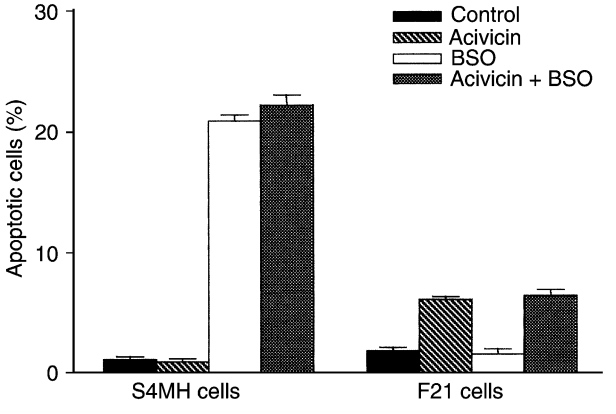

Figure 6. Apoptosis of S4MH and F21 cells exposed to BSO (100 μM), acivicin (1 μM) or BSO+acivicin. Apoptotic nuclei due to DNA fragmentation were detected by analyzing the reduced fluorescence of DNA-binding dye propidium iodide. Flow cytometry data are expressed as the percentage of apoptotic cells. All the experiments were repeated 3 times.

reduced the tumorigenic potential of S4MH cells (T/C of 57% on day 26), but no differences in tumor weights were found between the doses of 100 and 500 μM BSO (2.6 ± 0.1 and 2.6 ± 0.3 g, respectively). In the case of rats inoculated with F21 cells previously exposed to 500 μM BSO, the same degree of reduction (T/C of 57%; $p < 0.05$) in tumorigenic potential (0.4 ± 0.1 g) was found as in the rats inoculated with BSO-treated S4MH cells. In contrast, exposure to 100 μM BSO induced a significant increase (T/C value of 200%) in the tumorigenic potential of F21 cells (mean $\pm$ SD 1.4 ± 0.3 g).

Effects of *in vivo* BSO treatment on S4MH and F21 tumor growth

In an attempt to establish whether BSO administration *in vivo* would result in similar effects on the tumorigenic potential of tumor cells as when BSO

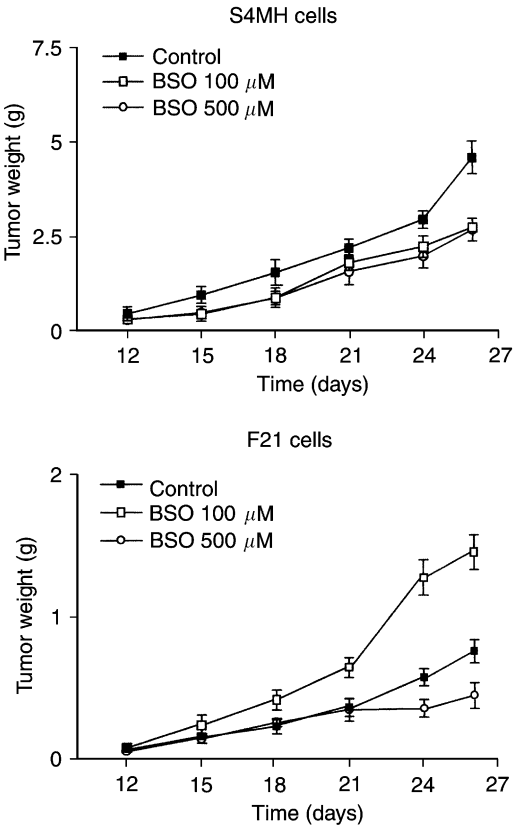

Figure 7. Tumorigenic potential of S4MH and F21 cells previously exposed *in vitro* to BSO. Culture cells were treated with 100 or 500 μM of BSO for 6 h and injected into rats 18 h after removing BSO from the culture medium. Tumors were evaluated 3 times per week for 4 weeks. Tumor weight was calculated by the following formula: $(L \times W^2)/2$, where L=longest axis and W=shortest axis. Data are expressed as tumor weight (g) and represent the mean $\pm$ SD of three experiments.

was administered *in vitro*, in a second series of experiments S4MH or F21 tumor-bearing rats were treated with 50 or 100 mg/kg of BSO. There was no significant weight loss or lethal toxicity with BSO treatment at any of the doses tested. As can be seen in Table 1, in S4MH tumor-bearing rats BSO treatment reduced tumor growth. Thus, on the day following treatment the T/C values registered were 43 and 37%, with doses of 50 and 100 mg/kg of BSO, respectively ($p < 0.05$), with the GDF values being 1.3 and 1.7, respectively. Whereas in F21 tumor-bearing rats treatment with 100 mg/kg of BSO produced no significant differences in tumor growth with respect to control (T/C of 91%), treatment with 50 mg/kg of BSO induced a significant reduction (1.9-fold with respect to the control) in the tumor-doubling time and, in consequence, a significant increase was found in tumor growth (T/C of 187%).

Discussion

GSH plays an important role in detoxification of a large number of electrophiles and, consequently, it can be a critical limiting factor in the response to treatment of malignancies. Although methods developed for measuring GSH in small biopsies of tumors[20] may have clinical usefulness in predicting relative resistance to chemotherapeutic drugs, the measurement of basal GSH levels could be insufficient to estimate the type of response that could be obtained when they are exposed to a therapy aimed at inhibiting GSH synthesis. Based on the results found in the present work, we think that the kinetics of GSH changes could be more suitable for monitoring tumor GSH metabolism and the response to a GSH therapy, as has also been suggested by other authors.[21] First, we observed that at the beginning of the cell culture there was no difference in the basal GSH levels between the poorly differentiated S4MH cells and the moderately differentiated F21 cells, whereas there was a different time course of GSH metabolism between these rhabdomyosarcoma

Table 1. Effect of *in vivo* BSO treatment on F21 and S4MH tumor growth

Tumor	Group	RTV[a]	T/C (%)[b]	*TD* (days)[c]	GDF[d]
S4MH	control	27.4 $\pm$ 4.7	–	1.1	–
	BSO 50 mg/kg	11.9 $\pm$ 1.3	43.4	2.5	1.3
	BSO 100 mg/kg	10.1 $\pm$ 2.6	36.8	3.0	1.7
F21	control	6.9 $\pm$ 1.2	–	4.3	–
	BSO 50 mg/kg	12.9 $\pm$ 3.6	186.9	2.3	–
	BSO 100 mg/kg	6.3 $\pm$ 1.0	91.3	4.8	0.1

[a]Expressed as the V_X/V_i index, where V_X represents the tumor volume on the day following treatment (day 26) and V_i is the volume of the same tumor at the start of treatment.
[b]RTV_{tr}/RTV_{con}, where RTV_{tr} represents the relative tumor volume of treated tumors and RTV_{con} that of untreated tumors.
[c]Tumor-doubling time.
[d]$(TD_{tr} - TD_{con})/TD_{con}$, where TD_{tr} is the tumor-doubling time of treated tumors and TD_{con} that of untreated tumors.

tumor cell lines. It has been previously reported that mitogen-induced proliferation is affected or regulated by GSH,[22,23] which is in accordance with the results obtained in the present study, where a good positive correlation between the time course of GSH levels and cell proliferation was observed. Thus, the S4MH cell line showed a significantly higher cell proliferation than the F21, which was in relation to a time-dependent greater increase of GSH content in these tumor cells. *In vivo*, the S4MH cell line also showed a significantly higher tumorigenic potential than the F21 cell line.

Secondly, S4MH cells were much more sensitive to BSO treatment than F21 cells. Thus, this inhibitor of γ-GCS produced a dose-dependent *in vitro* decrease of both GSH content and proliferation of S4MH cells, and also significantly reduced their tumorigenic potential *in vivo*. We showed previously that the specific inhibition of GSH synthesis by BSO decreased the adhesion efficiency of S4MH to endothelial cells and, in consequence, reduced their metastatic potential. Furthermore, we also demonstrated that BSO-induced GSH depletion produced an actin cytoskeleton disturbance which could explain the spherical shape adopted by BSO-treated S4MH cells that corresponds to a less differentiated phenotype.[24] In contrast, in BSO-exposed F21 cells no morphological changes were observed (cells exhibited the spindle shape characteristic of the phenotype differentiated), which is in consonance with the fact that only when BSO was administered at high doses was there a moderate reduction of GSH levels followed by a completely recovery. Moreover, treatment with BSO at low doses induced a significant increase in GSH levels and proliferation of F21 cells *in vitro*, and also increased their tumorigenic potential *in vivo*.

These findings indicate a more stable steady state of the GSH defense system in the F21 cell line than in the S4MH cells at a critical dose of BSO. The different BSO-induced effects observed between the two tumor cell lines can be explained by the fact that F21 cells had a significantly greater γ-GT activity than S4MH cells. Contradictory results have been described in relation to levels of γ-GT and tumor cell differentiation,[25,26] but this different γ-GT activity found between S4MH and F21 cell lines is in consonance with several other studies where increased γ-GT levels has been associated with differentiation.[27–29]

γ-GT is a membrane-associated ectoenzyme involved in the extracellular transport of GSH.[30] γ-GT can break the γ-glutamyl peptide bond of GSH and then the γ-glutamyl part can bind to cystine present in the medium; γ-glutamylcystine is transported into the cell where, through a transhydrogenation, it is reduced to γ-glutamylcysteine, which is a substrate for GSH synthetase. Thus, through this pathway the reaction catalyzed by γ-GCS can be bypassed and cellular GSH levels can now increase even in the presence of BSO.[31] Moreover, we found that inhibition of γ-GCS by BSO was followed by a significant increase in γ-GT activity in F21 cells, which therefore allows them not only to maintain GSH levels and to be viable to proliferate, but also to increase their GSH levels and their tumorigenic potential when this agent is administered at lower doses. These data agree with previous studies showing an increase of γ-GT activity after BSO exposure.[32] In contrast, the low γ-GT activity of S4MH cells impairs them for GSH synthesis *de novo* to maintain GSH levels when they are exposed to BSO. Since GSH is also involved in protein synthesis[2] and in cell survival,[33] GSH depletion and the consequent oxidative stress induced by BSO might be responsible for the nearly complete reduction of γ-GT activity and for the cell death via apoptosis observed in the BSO-treated S4MH cells. The important role of γ-GT in providing a substrate for GSH synthesis during oxidative stress[34] and in preventing apoptosis[35,36] has already been described. Indeed, inhibition of γ-GT by low doses of acivicin (a non-competitive inhibitor of γ-GT) is a sufficient stimulus for the induction of apoptosis in F21 cells and also for their sensitization to BSO treatment.

Conclusions

The results reported here in relation to the GSH metabolism differences between tumor cell lines with different degrees of differentiation and their different responses to BSO-induced inhibition of GSH synthesis suggest that the kinetics of GSH changes should give a more appropriate answer than the measurement of basal GSH levels. This fact could be important in the design of therapeutic strategies involving intracellular GSH level modification as a mechanism for tumor cell sensitization to cytostatic drugs.

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