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Effect of Glutathione Depletion on Inhibition of Cell Cycle Progression and Induction of Apoptosis by Melphalan (L-Phenylalanine Mustard) in Human Colorectal Cancer Cells

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ABSTRACT. Intracellular levels of glutathione have been shown to affect the sensitivity of cells to cell death-inducing stimuli, as well as the mode of cell death. We found in five human colorectal cancer cell lines (HT-29, LS-180, LOVO, SW837, and SW1116) that GSH depletion by L-buthionine-[*S*, *R*]-sulfoximine (BSO) below 20% of control values increased L-phenylalanine mustard (L-PAM; Melphalan) cytotoxicity 2- to 3-fold. Effects on kinetics of both cell cycle progression and cell death were further investigated in the HT-29 cell line. BSO treatment alone had no effect on cell cycle kinetics, but did enhance the inhibition of S phase progression as induced by L-PAM; at high concentration of of L-PAM, BSO pretreatment resulted in blockage in all phases of the cell cycle. Yet, BSO pretreatment did not affect the intracellular L-PAM content. L-PAM induced apoptosis in both normal and GSH-depleted cells. A combination of annexin V labeling and propidium iodide staining revealed that even the higher concentration of L-PAM (420 μM) did not induce apoptosis until 48 hr after treatment, but that induction of cell death was markedly accelerated as a result of GSH depletion: 48 hours after L-PAM (420 μM) treatment, GSH-depleted cells showed a 4-fold increase in DNA fragmentation and a 7-fold increase in the fraction of apoptotic (annexin V-positive) cells as compared to cells with normal GSH levels. Various antioxidant treatment modalities could not prevent this potentiating effect of GSH depletion on L-PAM cytotoxicity, suggesting that reactive oxygen species do not play a role. These data show that after BSO treatment the mode of L-PAM-induced cell death does not necessarily switch from apoptosis to necrosis. BIOCHEM PHARMACOL **58**;4:655–664, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. melphalan; glutathione; buthionine sulfoximine; apoptosis; cell cycle kinetics; HT-29 colorectal cancer cell line

GSH has many functions such as the reduction of disulfide linkages of proteins, reversible S-glutathionylation of cellular proteins, and the protection of cells against the effects of ROS§ that are formed during cellular metabolism [1, 2]. Moreover, GSH plays an important role in the metabolism of endogenous compounds such as 4-hydroxynonenal, as well as in the inactivation of many (reactive) xenobiotics, including certain anticancer drugs [3, 4]. Elevations in intracellular GSH have been associated with resistance to irradiation or chemotherapy, whereas reduction of GSH levels may increase the efficacy of these treatment modalities [5–10]. Drug resistance has been correlated with increased activity of glutathione *S*-transferases or γ-glutamyl cysteine synthetase, the rate-limiting biosynthetic step in GSH production. Transfection of the γ-glutamyl cysteine synthetase gene into tumor cells resulted in increased GSH levels and concomitant resistance to the alkylating drug L-PAM [11, 12]. It has been suggested that an enhanced rate of L-PAM detoxification by GSH conjugation is a major mechanism of drug resistance [13–15]. However, this was not substantiated by our earlier data: both in rats and humans, GSH conjugation played a very minor role in the hepatic elimination of L-PAM [16]. Thus, it is unlikely that modulation of GSH affects the rate of elimination of this drug, and there should be another explanation for the increased L-PAM sensitivity after GSH depletion.

Under normal conditions, cells maintain high levels of reduced GSH (1–20 mM). The ratio between GSH and its oxidized form (GSSG), i.e. the cellular redox potential,

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§ *Abbreviations:* BSO, L-buthionine-[*S*,*R*]-sulfoximine; $\Delta\psi$, mitochondrial membrane potential; Rh123, rhodamine 123; PI, propidium iodide; ROS, reactive oxygen species; NAC, N-acetyl-cysteine; FITC, fluorescein isothiocyanate; and L-PAM, L-phenylalanine mustard; melphalan.

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could directly modulate critical pathways in apoptosis [8, 17–20]. For instance, Marchetii *et al.* [21] recently suggested that this redox potential might affect the disruption of the inner mitochondrial transmembrane potential, possibly by affecting the redox state of apoptosis-regulating thiols resident in the mitochondrial membrane. A $\Delta\psi$ collapse is a common part of the apoptotic process preceding nuclear alterations and is probably mediated by mitochondrial megachannel opening (permeability transition) [22–26]. GSH may affect the apoptotic process at other levels as well [27, 28]. The GSH system represents a central and potent antioxidant system and it is widely accepted that ROS, as generated from mitochondrial electron transport systems, can stimulate the induction of apoptosis. Recently, Merad-Boudia *et al.* [27] showed that BSO treatment of neuronal cells caused a strong and transient increase in ROS, followed by decreased activities of complexes I, II, and IV of the respiratory chain, loss of mitochondrial function, and apoptotic cell death.

Whatever the mechanisms involved, one would expect that lowering the GSH levels would sensitize tumor cells to apoptosis-inducing stimuli; however, this is not always true. Fernandes and Cotter [5] observed that BSO treatment enhanced only the toxicity of alkylating compounds, with the mode of cell death switching from apoptosis to necrosis. A similar observation was reported with respect to GSH modulation and treatment with cisplatin [19]. From these findings, it was conjectured that the cellular redox state determines the type of cellular death induced. Nicotera and Leist [29] and Tsujimoto [30] have suggested that apoptosis and necrosis may represent only the extreme ends of a wide range of possible morphological and biochemical deaths; whereas these deaths may have some common upstream regulators, the end point would be largely determined by intracellular energy levels, many of the characteristic features of apoptosis requiring sufficient ATP generation. In this context, the above finding by Fernandez and Cotter [5] that treatment of several leukemia cell lines with alkylating agents such as L-PAM and chlorambucil led to a necrotic rather than apoptotic morphology in case of pretreatment with BSO would suggest that GSH depletion has a major impact on ATP generation, e.g. by reinforcing permeability transition to the extent that mitochondrial damage becomes incompatible with ATP production.

As part of a study on the role of GSH in the efficacy of L-PAM in treatment of colorectal cancer, we addressed the question as to whether GSH depletion could indeed affect the mode of cell death in this cell type and whether the basis for BSO-induced potentiation of toxicity of alkylating agents such as L-PAM is an increased generation of ROS. To this end, we tested a number of colorectal cancer cell lines and confirmed that BSO-induced reduction of cellular GSH levels below approximately 20% always led to a twofold increase in toxicity. We used one of the cell lines, HT-29, to investigate the effects of BSO and L-PAM on HT-29 cell cycle progression, as well as on the kinetics and mode of cell death.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The HT-29 human colon cancer cell line was cultured in McCoy's 5 A medium, whereas the LS-180, LOVO, SW1116, and SW837 cell lines were cultured in Dulbecco's modified Eagle's medium. All culture media were supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 μg/mL streptomycin, and 50 IU/mL penicillin (all obtained from GIBCO, Life Technologies).

GHS Analysis

Exponentially growing cells were washed with PBS and harvested by trypsinization. One part of the sample was used for GSH analysis and the remaining part for protein determination. GSH was extracted by adding 5% (w/v) aqueous sulfosalicyclic acid to pelleted cells. GSH in the cell extract was assayed by the colorimetric method of Ellman [31], with some previously reported modifications [32]. Total protein was determined by the method of Lowry *et al.* [33]. The GSH content was expressed as μmol per gram of protein.

Effect of BSO Exposure on GSH Content

To establish the effect of prolonged BSO (Sigma) treatment on intracellular GSH content, exponentially growing colorectal cancer cells were treated for 24 hr with medium containing various concentrations (0.2–1250 μM) BSO. After the 24-hr incubation period, the cells were treated and analyzed for GSH as described above. All experiments were performed at least in triplicate.

Cytotoxicity Assessment

Cells were seeded at 1000 (HT-29), 2000 (LS-180, LOVO, and SW837) or 4000 (SW1116) cells/well (96-well plate) in 100 μL medium. The differences in initial cell density are related to differences in growth rate of the 5 cell lines: at the end of the experiment 6 days later, we needed a sufficient quantity of each cell line to allow assessment of cytotoxicity. After a 24-hr incubation period, another 100 μL medium with or without BSO was added to the existing medium. Another 24 hr later, the cells were washed and treated with various concentrations of L-PAM (Wellcome Pharmaceuticals). They were incubated with L-PAM for 1 hr, washed twice and new medium was added. After an additional 3-day period, new cell tissue culture medium was added. Cell viability was determined by the WST-1 assay (Boehringer) at day four after L-PAM treatment. WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) is an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)-like tetrazolium-based colorimetric assay [34] that is used to measure cell survival and chemosensitivity. To evaluate whether an increase in the antitumor efficacy of L-PAM by GSH

depletion could be diminished by treatment with iron chelators and/or antioxidants, HT-29 cells were plated and treated as described above, but during the 1-hr L-PAM (52 μM) treatment, one of the following was added: 10 μM diphenyl-*p*-phenylendiamine, 100 μM desferal, 100 μM ascorbic acid, 1 mM GSH, or 1 mM NAC (all from Sigma). All experiments were performed at least in triplicate.

Flow Cytometric Cell Cycle Changes

To determine if GSH depletion modifies the pattern of L-PAM-induced changes on the cell cycle, HT-29 cells with or without BSO pretreatment were exposed to 52 and 420 μM L-PAM. HT-29 cells were seeded in tissue culture flasks and 25 μM BSO was added after a 24-hr attachment period. After an additional 24 hr, both BSO-treated cells and control cells were exposed to L-PAM for 1 hr, after which fresh medium was added to the cells. At days 1 and 3 after L-PAM treatment, attached (after trypsinization) and detached cells were combined. Suspensions of single nuclei were prepared for flow cytometry, stained with PI, and analyzed on a FACScan flow cytometer (Becton Dickinson) as described by Vindelov *et al.* [35].

Measurement of L-PAM in Control and GSH-Depleted Cells

Exponentially growing HT-29 cells were incubated with 25 μM BSO for 24 hr. Both BSO pretreated and control cells were then exposed to 52 and 420 μM L-PAM. After a 1-hr incubation with L-PAM, cells were washed twice with PBS and harvested (by trypsinization). L-PAM was extracted by adding 5% (v/v) aqueous $HClO_4$ to pelleted cells. The L-PAM concentration in the cell lysate was measured by an HPLC assay [36], and the L-PAM content was expressed as nmol/10^6 cells.

Detection of DNA Fragmentation

HT-29 cells (0.25×10^5/well) were seeded in 6-well plates and after a 24-hr attachment period, 25 μM BSO was added. After an additional 24 hr, cells were washed and both BSO-treated and control cells were exposed to 420 μM L-PAM for 1 hr. After L-PAM incubation, fresh medium was added to the cells. Detached and attached cells (after trypsinization) were combined following collection 48 hr after L-PAM treatment, and DNA fragmentation was analyzed using the *in situ* cell death detection ELISA^{plus} test (Boehringer). This assay is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. Briefly, after washing with PBS, the cells were counted and 10,000 cells were resuspended in lysis buffer (provided in the kit). DNA fragmentation was analyzed in the cell lysates according to the manufacturer's instructions. An enrichment of nucleosomes in the cytoplasm of treated cells was calculated by dividing the absorbance (A_{405nm}/A_{490nm}) of L-PAM-treated cells by the absorbance of the corresponding controls.

Detection of Phosphatidylserine Using Annexin V–FITC and Measurement of Δψ

HT-29 cells (0.25×10^5/well) were seeded in 6-well plates and after a 24-hr attachment period, 25 μM BSO was added. After an additional 24 hr, cells were washed and both BSO-treated and control cells were exposed to 420 μM L-PAM for 1 hr. Detached and attached cells (after trypsinization) were combined following collection 48 and 96 hr after L-PAM treatment, and cell death was analyzed using the APOPTEST™-FITC kit (Nexins Research). Briefly, peletted cells were resuspended in culture medium containing 1.5 mM $CaCl_2$, and after a 1-hr acclimatization period at room temperature, the cell sample was incubated with FITC-conjugated annexin V and PI (Sigma) for 10 min (according to the manufacturer). Samples were measured on a FACScan flow cytometer, and four distinct populations were identified as annexin V-negative and PI-negative (R1; −/−; vital), annexin V-positive and PI-negative (R2; +/−; apoptotic), annexin V-positive and slightly PI-positive (R3; +/±; late apoptotic), and annexin V-positive and PI-positive (R4; +/++; necrotic). Δψ of cell samples obtained 24 and 48 hrs after L-PAM or BSO/L-PAM treatments were determined by flow cytometry as previously described [37] using Rh123.

RESULTS

Effect of GSH Depletion on L-PAM Cytotoxicity

The GSH levels of all five colorectal cancer cell lines were substantially decreased after a 24-hr exposure to 5 μM BSO and higher concentrations (Fig. 1). This GSH depletion enhanced cytotoxicity of L-PAM as measured by the WST-1 assay. For instance, a 24-hr pretreatment of HT-29 cells with 25 μM BSO decreased the GSH content to 19% of control value and enhanced the cytotoxicity of L-PAM 2.7-fold (Fig. 2, Table 1). Interestingly, potentiation of L-PAM cytotoxicity was only observed when GSH was depleted by more than 75–80% (Fig. 3). Therefore, in all subsequent experiments we used BSO concentrations sufficient to cause a drop in GSH content below 20–25% of control values. Depending on the cell lines investigated, these levels were reached by treatment with BSO concentrations ranging from 25–1250 μM, although the increases in cytotoxicity were all within a remarkably narrow range of 2.2–2.7 (Table 1). Further experiments focused on the effects of BSO treatment on the kinetics of cell proliferation and cell death after L-PAM treatment in the HT-29 cell line, a frequently used cell line in this type of study.

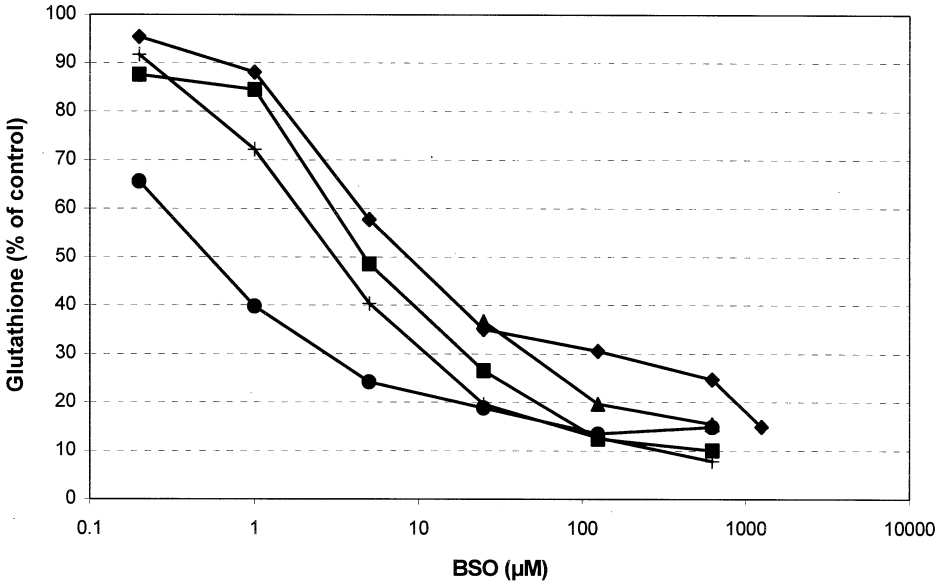

FIG. 1. Effect of a 24-hr BSO treatment on cellular GSH in 5 human colon cancer cell lines: HT-29 (●), LOVO (■), LS-180 (◆), SW837 (▲), and SW1116 (+). GSH was assayed at the end of the 24-hr treatment period.

Effect of GSH Depletion on L-PAM-Induced Cell Cycle Changes

Because differences in cell cycle (i.e. cellular DNA content) distribution tend to be a sensitive indicator for the efficacy of anticancer drugs, we compared flow cytomeric DNA histograms obtained from untreated, BSO-treated, L-PAM-treated, and BSO/L-PAM-treated HT-29 cells at 24 and 72 hr after treatment. Cell monolayers with or without BSO pretreatment (25 μM) were treated for 1 hr with either a low (52 μM) or high (420 μM) L-PAM concentration, and cells (combined detached and attached) were harvested at 24 hr and 72 hr after L-PAM treatment. As shown in Fig. 4, DNA distribution patterns of BSO only-treated cells were identical to controls, indicating that depletion of GSH by itself did not affect cell cycle progression in this cell line. Cells treated with 52 μM L-PAM showed accumulation in late S phase at 24 hr, whereas at 72 hr the DNA histogram showed a marked accumulation of material with a pre-$G_{1/0}$ DNA content, indicative of cellular debris. However, the latter histogram also showed a normalization of distribution of cells with a $G_{1/0}$, S, and G_2/M DNA content respectively, suggesting that part of the cell population survived L-PAM treatment. Pretreatment of cells with BSO resulted in an accumulation of cells in mid S phase at 24 hr and a disappearance of cells with $G_{1/0}$ DNA content; accumulation of cells with a G_2/M DNA content occurred at 72 hr, indicating that most, if not all, cells had died (or were in the process of dying). An even more dramatic effect on cell cycle progression was obtained by treatment with the high dose of L-PAM: cells accumulated in early S phase and the absence of a G_2/M peak at 72 hr indicate a total absence of progression through the S phase. Surprisingly, after GSH depletion, cells treated with 420 μM L-PAM at 24 hr showed a normal DNA distribu-

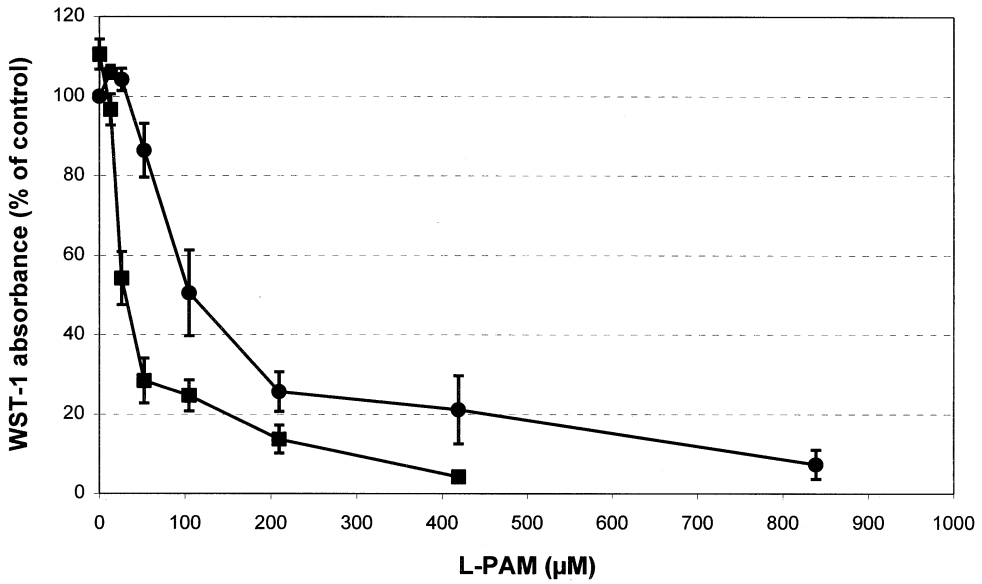

FIG. 2. Cytotoxicity of a 1-hr L-PAM incubation of normal (●) and GSH-depleted HT-29 cells (■) (which had been pretreated for 24 hr with 25 μM BSO), as measured by WST-1 absorbance 96 hr after L-PAM treatment. Data points represent means ± SD (N = 4).

TABLE 1. Effect of BSO pretreatment on the cytotoxicity of melphalan in five human colorectal cancer cell lines

Cell line	BSO (μM)	GSH (% of control)	I.C. 90 melphalan (μM) control cells	I.C. 90 melphalan (μM) GSH-depleted cells	Dose-modifying factor
HT-29	25	18.8 ± 8.0	717 ± 141	278 ± 62	2.7 ± 0.6
LOVO	125	12.4 ± 5.1	53 ± 10	22 ± 6	2.4 ± 0.2
LS-180	1250	14.9 ± 3.5	521 ± 162	236 ± 102	2.3 ± 0.3
SW837	125	19.7 ± 5.6	741 ± 52	312 ± 36	2.4 ± 0.4
SW1116	25	19.6 ± 3.4	665 ± 97	298 ± 37	2.2 ± 0.0

For each cell line, a BSO concentration was chosen that caused a drop in GSH content to at least 20% of control value (See Fig. 1). The GSH content (expressed as % of control, i.e. *not* treated with BSO) represents the level of GSH directly before melphalan treatment. Cytotoxicity (I.C. 90) is expressed as the concentration of melphalan that led to a drop in WST-1 absorbance to 10% of control value. The dose-modifying factor is calculated by dividing the I.C. 90 melphalan of control cells by that of BSO-pretreated cells. The control GSH levels were: HT-29, 28.7 ± 8.8 μmol/g protein; LOVO, 26.8 ± 2.1 μmol/g; LS-180, 36.8 ± 3.5 μmol/g; SW837, 42.4 ± 8.3 μmol/g; and SW1116, 32.5 ± 1.9 μmol/g. Values represent means ± SD.

tion, which had almost disappeared at 72 hr. This indicates a total block of both DNA synthesis and mitosis and suggests that cells died within all cell cycle compartments.

Effect of BSO Pretreatment on L-PAM Content of HT29 Cells

In order to verify whether BSO treatment might enhance the cellular uptake of L-PAM, concentrations were measured in both normal and GSH-depleted HT-29 cells. After a 1-hr incubation with 52 μM L-PAM, the amount of L-PAM in control cells was 0.03 ± 0.02 nmol/10^6 cells versus 0.04 ± 0.01 nmol/10^6 cells in GSH-depleted cells; in the case of 420 μM L-PAM, it was 0.38 ± 0.02 nmol/10^6 cells versus 0.39 ± 0.1 nmol/10^6 cells in GSH-depleted cells. Therefore, these data show that L-PAM uptake is not influenced by GSH depletion.

Effects of GSH Depletion on L-PAM-Induced Cell Death

To establish the mode of cell death by L-PAM treatment, cell samples obtained 48 hr after a 1-hr treatment with 420 μM L-PAM were analyzed for DNA fragmentation, using the cellular DNA fragmentation Elisa^{plus} test (Boehringer), which allows determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Presence of nucleosomes in the cytoplasm is indicative of DNA fragmentation. Nucleosome levels in BSO-, L-PAM-, and BSO/L-PAM-treated cells were 1.5, 3.5, and 14.0 times the non-treated control levels (statistically significantly different from control or L-PAM alone, $P < 0.05$), indicating that BSO/L-PAM treatment resulted in a fourfold higher level of cellular DNA fragmentation compared to L-PAM treatment alone.

To investigate the kinetics of cell death, cell monolayers with or without a 24-hr 25 μM BSO pretreatment were treated for 1 hr with 420 μM L-PAM. Forty-eight and ninety-six hours after L-PAM treatment, both detached and attached cells were collected and analyzed with FITC-labeled annexin V, which strongly binds to phospholipid phosphatidylserine (PS) on the surface of cell membranes. PS is normally present in the inner leaflet of the lipid bilayer of the plasma membrane, but rearranges during apoptosis such that it can be detected at the outer leaflet of the plasma membrane. After incubation of the HT-29 cells with annexin V–FITC and PI, four distinct populations

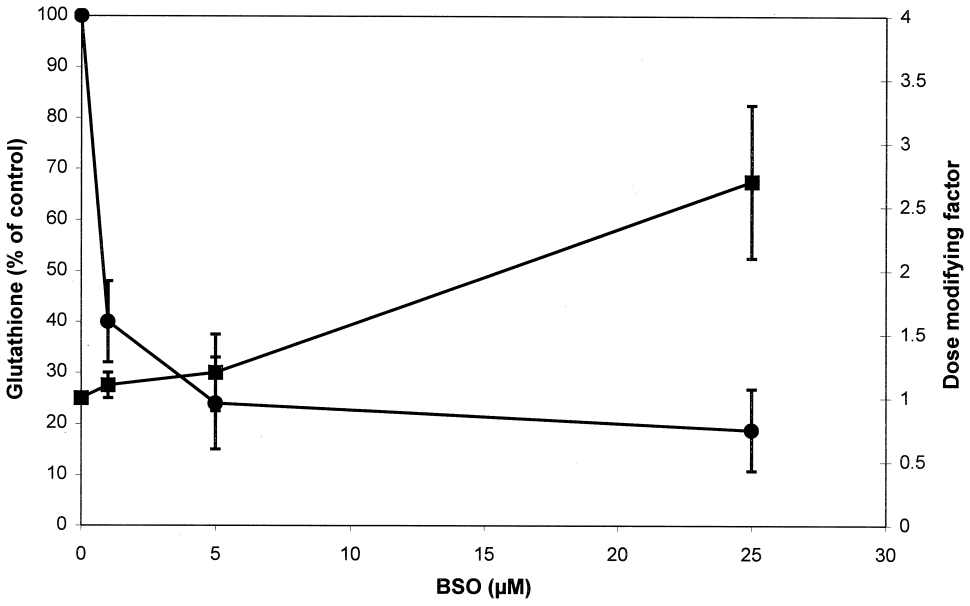

FIG. 3. Effect of a 24-hr BSO pretreatment on the GSH content (●) and L-PAM cytotoxicity of HT-29 cells. The dose-modifying factor (■) was determined by dividing the I.C. 90 (90% inhibitory concentration as measured by WST-7 absorbance) of L-PAM in untreated cells by that in the BSO-pretreated cells. Data points represent means ± SD (N = 3).

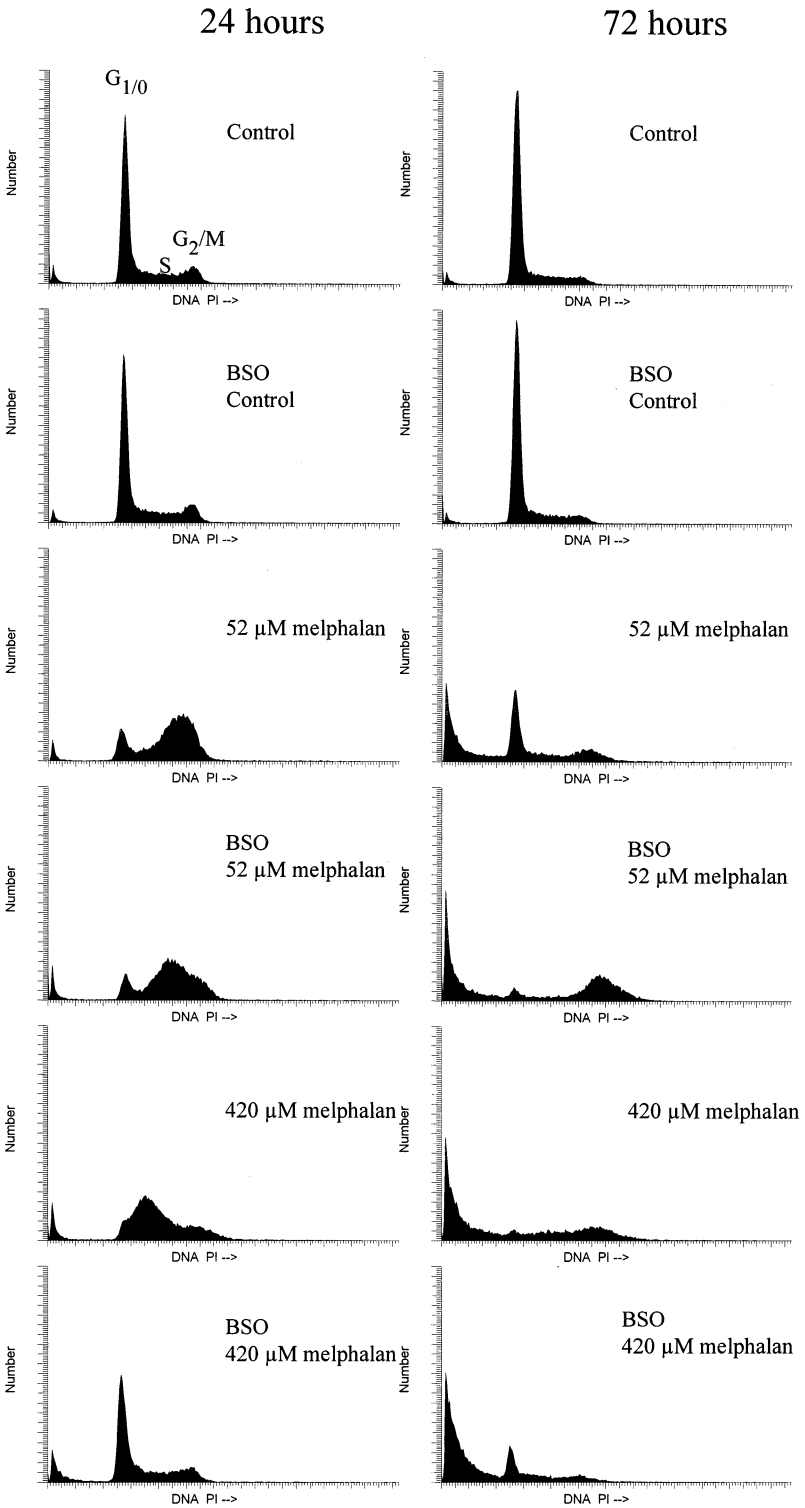

FIG. 4. Flow cytometric DNA histograms of control and GSH-depleted cells (which had been pretreated for 24 hr with 25 μM BSO) obtained 24 and 72 hr after a 1-hr (52 or 420 μM) L-PAM treatment. Both attached and detached cells were collected, and the DNA content of the cells was analyzed using flow cytometry.

were observed on the flow cytometric histogram: annexin V-negative/PI-negative (−/−; vital), annexin V-positive/PI-negative (+/−; apoptotic), annexin V-positive/slightly PI-positive (+/±; late apoptotic), and annexin V-positive/strongly PI-positive (+/++; necrotic). The +/± population probably represents late apoptotic cells, because these cells were annexin V-positive with a low PI fluorescence (Fig. 5), which could be due to loss of DNA [38].

In controls, most cells were viable: only 9–13% was recovered in the three annexin V-positive categories. BSO (25 μM) alone did not change this pattern. Treatment with 420 μM L-PAM did not show changes at 48 hr after treatment, but at 96 hr a significant proportion of the cells had died: compared to untreated control cells, the percentage of apoptotic cells (both categories together: +/− and +/±) had increased from 7% to 26%, while the percentage

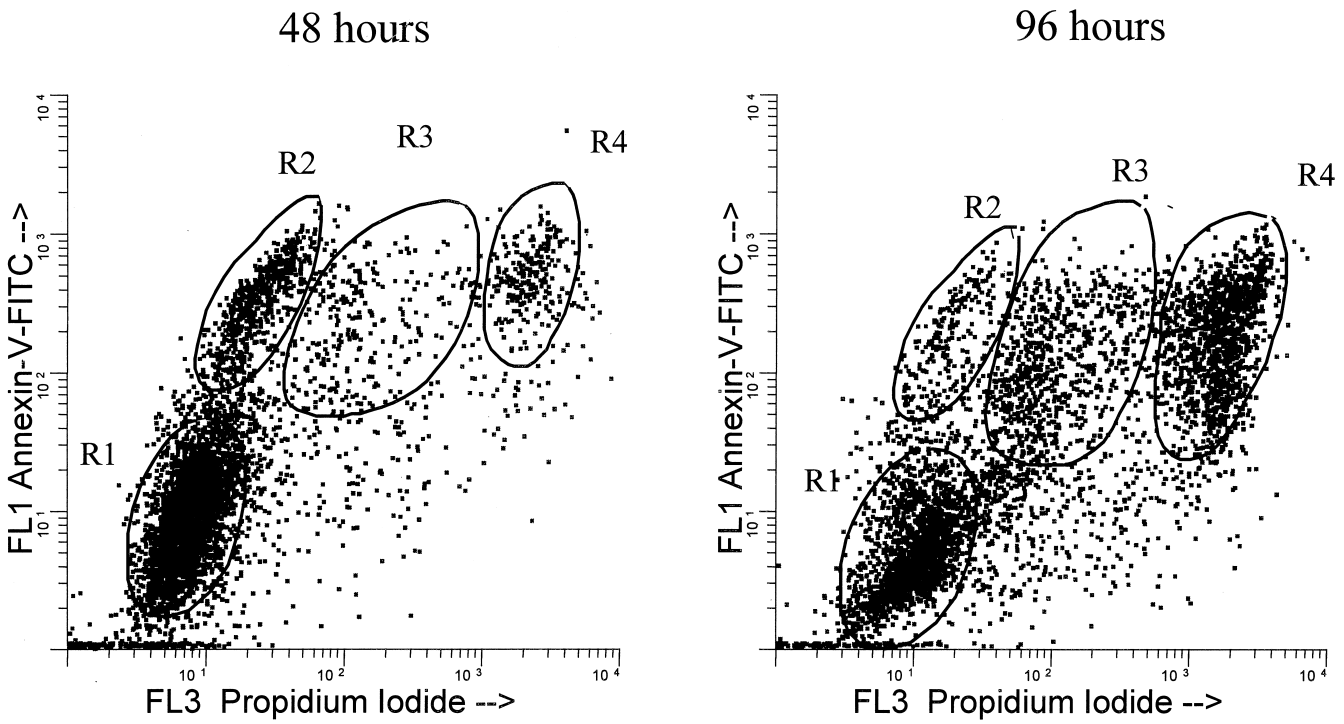

FIG. 5. Flow cytometric dot-plot of annexin V–FITC fluorescence versus PI fluorescence of HT-29 cells 48 and 96 hr after BSO + L-PAM (420 μM) treatment. The dot-plot shows four distinct populations: vital (R1; annexin V–FITC-negative and PI-negative), apoptotic (R2; annexin V–FITC-positive and PI-negative), late apoptotic (R3; annexin V–FITC-positive and slightly PI-positive), and necrotic cells (R4; annexin V–FITC-positive and strong PI-positive).

of necrotic cells increased from 6% to 15% (Table 2). Evidently, development of apoptotic cell death by L-PAM required at least two days in this cell line. When L-PAM was tested in BSO-treated cells, however, its cytotoxic effect was strongly increased. Already 48 hr after L-PAM treatment, the percentage of apoptotic cells had increased from 5% in L-PAM only to 18% in L-PAM/BSO-treated cells. The necrotic category did not yet show an increase, suggesting that necrosis may follow apoptosis in time ('secondary necrosis'). At 96 hr, the percentage of +/− (apoptotic) cells had not changed, whereas the percentage of +/++ (necrotic) cells had increased from 15% to 25% (Table 2).

Effect of L-PAM on Δψ

It is generally believed that one feature of apoptotic cell death is a collapse of the mitochondrial membrane potential. To measure the effect of L-PAM with respect to changes in Δψ, cells were harvested 24 and 48 hr after a

TABLE 2. Effect of melphalan on annexin V binding and PI uptake in HT-29 cells

Treatment	Vital (%)	Apoptotic (%)	Late apoptotic (%)	Necrotic (%)
48 hr after melphalan treatment				
Control	91.2 ± 0.7	2.0 ± 0.5	1.9 ± 0.3	4.9 ± 0.8
Control + BSO 25 μM	89.4 ± 1.4	2.1 ± 0.4	3.2 ± 0.4	5.4 ± 1.0
Melphalan	87.5 ± 3.3	1.7 ± 0.1	3.3 ± 1.3	7.6* ± 2.1
Melphalan + BSO 25 μM	73.2† ± 1.1	11.3† ± 1.3	7.2† ± 0.5	8.3* ± 2.0
96 hr after melphalan treatment				
Control	87.1 ± 1.1	2.2 ± 0.5	4.9 ± 0.9	6.0 ± 1.2
Control + BSO 25 μM	84.7 ± 1.7	2.6 ± 0.2	5.9 ± 0.7	6.8 ± 1.4
Melphalan	58.3* ± 3.7	8.4* ± 0.6	18.2* ± 0.6	15.2* ± 2.9
Melphalan + BSO 25 μM	46.4† ± 3.2	7.8* ± 0.2	20.6† ± 1.2	25.5† ± 2.9

Attached and detached cells of a control or GSH-depleted (pretreated for 24 hr with 25 μM BSO) HT-29 cell line were collected 48 and 96 hr after a 1-hr 420 μM melphalan treatment. Cells were labeled with annexin V–FITC and PI and analyzed by flow cytometry. Four cell populations were defined as in legend to Fig. 5. Data points represent means ± SD (N = 3).

*Statistically different from control.

†Statistically significant from control and melphalan only-treated cells; one-way ANOVA, LSD (least significant difference) procedure, $P < 0.05$.

TABLE 3. Effect of GSH depletion on melphalan-induced changes in mitochondrial membrane potential ($\Delta\psi$) of HT-29 cells

Treatment	$\Delta\psi$ 24 hr after treatment (% of control)	$\Delta\psi$ 48 hr after treatment (% of control)
Control	100	100
Control + BSO 25 μM	98 ± 1	100 ± 4
Melphalan	126* ± 2	206* ± 12
Melphalan + BSO 25 μM	103 ± 2	152† ± 3

At 24 and 48 hr after a 1-hr 420 μM melphalan incubation of both control and BSO (25 μM, 24 hr) pretreated cells, both attached and detached cells were collected, mixed and $\Delta\psi$ was measured using flow cytometry. Shown are the means ± the SD (N = 3).

*Significantly different from both control and melphalan + BSO treatment (one-way ANOVA, LSO [least significant difference] procedure; $P < 0.05$).

†Significantly different from other treatments.

1-hr 420 μM L-PAM treatment and stained with Rh123 and PI. Surprisingly, an increase in Rh123 fluorescence was observed both 24 and 48 hr after L-PAM treatment (Table 3). Whereas BSO by itself did not seem to affect Rh123 fluorescence, in combination with L-PAM it did appear to reduce the enhancement of Rh123 fluorescence.

Effect of Antioxidant Treatments on the L-PAM Toxicity-Potentiating Effect of GSH Depletion

To evaluate whether ion chelators and/or antioxidants are able to reduce the potentiating effect of GSH depletion on L-PAM cytotoxicity, 100 μM desferal, 10 μM diphenyl-*p*-phenylendiamine, 100 μM ascorbic acid, 1 mM GSH, or 1 mM NAC was added to GSH-depleted HT-29 cells (pretreated with 25 μM BSO for 24 hr) during the 1-hr L-PAM (52 μM) treatment. No significant protection of L-PAM-induced cell death by any of these compounds was observed (data not shown). Even the addition of GSH and NAC (prolonged incubation with the other scavengers was too toxic by itself) during and for 3 days after L-PAM treatment did not significantly protect against L-PAM-induced cell death.

DISCUSSION

In this study we demonstrate, in 5 human colorectal cancer cell lines growing as monolayers, that pretreatment with the GSH synthesis inhibitor BSO more than doubled L-PAM cytotoxicity, but only when GSH was depleted below approximately 20–25% of the control level. Under the experimental conditions used, the BSO concentration required to reach this threshold ranged a factor of 50 (25–1250 μM) among the different cell lines, possibly reflecting differences in their GSH homeostasis mechanisms. A recent study on BSO pharmacokinetics in cancer patients revealed that after a 30-min infusion of 13.1 g/m^2 BSO the peak concentration was 7250 μM; after 6 hr this level was decreased to 670 μM, indicating that in humans effective BSO concentrations can indeed be obtained for a prolonged period of time [39, 40].

In an attempt to reveal the cellular mechanism(s) by which GSH depletion enhanced L-PAM toxicity, we further investigated the effects of BSO/L-PAM on cell cycle kinetics and mode of cell death using the HT-29 cell line. BSO treatment alone had no effect on cell cycle progression, but resulted in a marked shift in L-PAM-induced cell cycle blockage from G_2/M/late S phase to mid S phase (52 μM L-PAM) and from early S phase to the G_1 phase (420 μM L-PAM). Several studies have shown that at low L-PAM concentrations a transient accumulation of cells in the G_2/M phase occurred whereas an irreversible S phase block was observed at higher L-PAM concentrations [41, 42]. The fact that we did not observe a different cellular L-PAM content as a result of GSH depletion would imply that (a) L-PAM is not readily detoxified via GSH conjugation (which is supported by our previously published data from isolated liver perfusions with L-PAM in humans [16]) and (b) that GSH depletion interferes either with the cellular activity of L-PAM itself or with the response of the cell to this compound. This response may involve attempts to repair the inflicted DNA damage [43], the signal transduction that leads from damage to induction of apoptosis, or the execution of the apoptotic process itself. In this context, it might be relevant that GSH is not only sequestered into mitochondria, but that there may also be a GSH pool in the nucleus; it has been conjectured that the nuclear redox defined by nuclear GSH concentrations may have pleiotropic effects on susceptibility to DNA damage, gene transcription, and nuclear signal transduction both before and following an apoptotic stimulus [44].

In the HL-60 promyelocytic leukemia and U937 monoblastoid cell lines, L-PAM cytotoxicity was reported to be increased by GSH depletion, but with the mode of cell death changing from apoptosis to necrosis [5]. Similar results have been published for treatment of a human pharyngeal carcinoma cell line with cisplatin, and it was proposed that reduction in GSH levels in fact may inhibit the induction and/or execution of the apoptotic process [19]. It should be noted, however, that in the HL-60 and U937 cells, apoptosis/necrosis was evaluated 4 hr after L-PAM treatment, whereas in HT-29 cells the interval of onset of apoptosis was measured in days rather than hours. As compared to L-PAM only-treated HT-29 cells, GSH depletion before L-PAM treatment (420 μM) translated into a 4-fold increase in DNA fragmentation and a 6.6-fold increase in the number of annexin V-positive cells at 48 hr, without any increase in cells strongly stained by PI, i.e. 'necrotic' cells. A likely explanation would be that GSH depletion accelerated the induction of L-PAM-induced apoptosis and that the observed increase in strong PI staining at 96 hr is probably the consequence of the fact that in tissue culture the majority of apoptotic cells are not phagocytozed, but are subjected to a process known as 'secondary necrosis'.

Apoptosis in many cases appears to be triggered by

permeability transition, which allows the free distribution of solutes of <1.5 kDa and some proteins across the mitochondrial membrane, thereby leading to disruption of the electrochemical gradient across the inner membrane [45,46]. When we examined the fluorescence of the membrane-permeant, cationic dye Rh123 at 48 hr after L-PAM treatment, we observed an increase instead of a decrease relative to control values. Recently, vander Heiden *et al.* [47] reported a similar increase in Rh123 fluorescence in Jurkat T cells 6 hr after treatment with staurosporine or anti-Fas antibodies and in FL5.12 cells 18 hr after interleukin-3 deprivation. They argued that, according to the Nernst equation, Rh123 fluorescence is not only dependent on $\Delta\psi$, but also on the volume of the mitochondria. Their electron microscopy data provided evidence for swelling of the space between the inner and outer mitochondrial membranes, occurring prior to nuclear chromatin condensation; experiments with a protonophore revealed an H^+ ion-independent increase in fluorescence, reflecting mitochondrial swelling. Thus, swollen mitochondria, believed to be a hallmark of necrosis, can also occur during the induction phase of apoptosis, prior to cytochrome *c* release from the mitochondria, caspase activation, and changes in nuclear morphology.

Recently, another mechanism for the cytoprotective effect of GSH was described, which involved the scavenging of lipid peroxidation products generated by a nitrogen mustard, mechlorethamine, in isolated hepatocytes [48]; the addition of radical scavengers or antioxidants prevented the increase in cytotoxicity of this nitrogen mustard as a result of GSH depletion. Antioxidants did not protect our HT-29 cells from the BSO-induced enhancement of L-PAM toxicity. Recently, Liu *et al.* [49] reported that sulfur-containing antioxidants such as NAC could be very strong inducers of p53 expression, leading to apoptotic cell death in several transformed cell types. However, because HT-29 cells lack functional p53, the latter phenomenon could not explain the lack of protection by NAC. We therefore tentatively conclude that in HT-29 cells, ROS are probably not an important factor in the L-PAM toxicity-potentiating effect of BSO.

In summary, in all human colorectal cancer cell lines tested, GSH depletion by clinically relevant BSO concentrations increased L-PAM cytotoxicity approximately 2- to 3-fold, but this effect was not diminished by radical scavengers or antioxidants. Cell kinetic analysis of one of these cell lines revealed an accumulation of late S phase cells after L-PAM treatment. GSH depletion by BSO alone did not have any impact on cell cycle progression, nor did it affect cellular L-PAM levels, although it did shift the L-PAM-induced cell cycle blockage towards an earlier part of S phase. In contrast to reports showing that GSH depletion changes the mode of drug-induced cell death to a necrotic phenotype, we observed an earlier onset of apoptotic cell death. Therefore, rather than having an effect on detoxification mechanisms of L-PAM, BSO pretreatment most likely increased the sensitivity of colorectal cancer cells to the induction of apoptosis.

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