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APOPTOSIS OR NECROSIS: INTRACELLULAR LEVELS OF GLUTATHIONE INFLUENCE MODE OF CELL DEATH

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Abstract—The effects of lowering intracellular glutathione (GSH) concentrations on the toxicity of alkylating agents, an RNA synthesis inhibitor and topoisomerase 1 and 2 inhibitors to a number of human leukaemic cell lines were evaluated. By using the GSH synthesis inhibitor DL-buthionine-(*S,R*)-sulfoximine (BSO), GSH levels were artificially reduced. Cells with low GSH concentrations were exposed to a number of cytotoxic agents and the resultant mode of cell death was analysed using morphological and biochemical criteria. It was found that untreated cells exposed to the above drugs underwent apoptosis to varying extents. However, the toxicity of alkylating agents was dramatically increased to all cell lines on lowering GSH levels, with the mode of cell death switching from apoptosis to necrosis. The reduction of GSH levels had no effect on the toxicity of actinomycin-D, camptothecin or etoposide, nor did it affect the mode of cell death induced by these agents. These observations suggest that modulation of GSH levels effect the toxicity of alkylating agents and that GSH influences the mode of cell death induced by alkylating agents.

Key words: apoptosis; GSH; alkylating agents

Cell death under physiological conditions usually occurs via apoptosis. This mode of cell death, first characterized by Kerr *et al.* [1], allows the cell to control its own demise. The process of "self" regulated death involves a series of morphological changes including cell shrinkage, nuclear condensation, nuclear fragmentation and zeiosis [2]. Zeiosis is the fragmentation of the cell into membrane bound "apoptotic" bodies, which are then ingested by surrounding phagocytic cells [3]. This prevents tissue damage by lysosomes and other degrading substances released by the dying cell. In many cell systems the morphological alterations of apoptosis are accompanied by endonuclease mediated nucleosomal fragmentation of the chromatin [4, 5]. This enzymatic cleavage at the DNA linker region, has long been regarded as a biochemical hallmark of apoptosis, although recent evidence suggests that some types of apoptosis may occur in the absence of DNA fragmentation [6, 7].

From a pharmacological viewpoint, this mode of cell death has received much attention, since it has been found that many chemotherapeutic drugs induce apoptosis in a variety of tumour cell lines [8]. However, the ability of these drugs to induce cell death is often limited by the development of drug resistance in the malignant cell. An example of this being the increased expression of *p*-glycoprotein which may enhance the efflux of chemotherapeutics out of the cell [9]. Increased attention has focused on the role of GSH† and GSH-related enzymes in drug resistance, as GSH (a tripeptide thiol) has been implicated in the resistance to oxygen radicals, various endogenous toxins, alkylating agents and other chemosensitizers [10, 11]. Alkylating agents are capable of inducing apoptosis in a number of malignant lines [12, 13], and as GSH levels appear greater in many neoplasms compared to normal tissue [14], it is reasonable to assume that cell resistance to apoptosis induced by these agents may involve GSH. However, it should be noted that, in other cell lines, the importance of GSH metabolism in drug resistance has been questioned. Work by Campling *et al.* [15], found very few significant correlation's between levels of GSH and drug sensitivity in various human small cell lung cancer cell lines. Thus the role of glutathione in cell defence mechanisms may vary between cell lines.

An obvious method of circumventing various mechanisms of cell resistance, such as GSH-induced resistance to alkylating agents, is to increase the levels of drug used. However, simply increasing drug concentrations can affect the mode of cell death, as studies from our laboratory have shown. As one increases the concentration of cytotoxic agents the mode of cell death changes from apoptosis to necrosis [12], a mode of cell death which can lead to further tissue damage due to the release of degradative enzymes from the dying cell. The question being addressed in this study is, if we artificially reduce GSH levels in cells will we see an increase in the levels of drug mediated cell death and will this be via apoptosis or necrosis? This is of interest because it is more desirable to kill cells via apoptosis rather than necrosis which is associated with a destructive inflammatory response reaction.

In the present study we show that decreasing the

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† Abbreviations: DTNB, 5,5-dithio-*bis*(2-nitrobenzoic acid); BSO, DL-buthionine-(*S,R*)-sulfoximine; SSA, sulphosalicylic acid; GSH, glutathione.

intracellular levels of GSH markedly enhances the cytotoxicity of alkylating agents, but the mode of cell death switches from apoptosis to necrosis.

MATERIALS AND METHODS

Reagents. DTNB, BSO, melphalan, chlorambucil, actinomycin-D, camptothecin, etoposide, triethanolamine–HCL, EDTA, NADPH, GSH and GSH reductase were all purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Sodium dihydrogen phosphate (NaH_2PO_2) and di-sodium hydrogen phosphate (Na_2HPO_4) were obtained from Riedel-de Haen AG (Seelze, Hannover, Germany) and sulphosalicylic acid was provided by BDH Ltd (Poole, U.K.).

Stock solutions (6 mM) of DTNB and NADPH (0.3 mM) were prepared in 125 mM phosphate buffer (162 mM NaH_2PO_4, 125 mM Na_2HPO_4, 6.3 mM EDTA), pH 7.5. Stock solutions of sulphosalicylic acid and triethanolamine–HCl were prepared in distilled water. Both melphalan and chlorambucil were dissolved in 10% ethanol containing 0.16% HCl. Actinomycin-D, camptothecin and etoposide were dissolved in DMSO. A 1 mg solution of BSO was prepared in RPMI-1640. GSH standards were prepared fresh each time, 1 mg/mL GSH was made up in 3% SSA and 15 μL was added to 985 μL of 3% SSA to give a final concentration of 15 μg/mL.

Cell lines. Human cell lines HL-60 [16], a promyelocytic leukaemia line; U937 [17], a monoblastoid line and K562 [18], a chronic myelogenous leukaemia line were maintained at 5×10^6 cells/mL in RPMI-1640 (Gibco Ltd, Paisley, U.K.) to which was added 10% foetal calf serum (Biochrom KG, Germany). Cells were maintained at 37° in a 5% CO_2, 95% air incubator.

Determination of total intracellular GSH levels. Cells were harvested from culture by centrifugation at 200 *g* for 5 min. The cells were washed twice in PBS (0.1 M phosphate-buffered 0.15 M NaCl, pH 7.5) and lysed in 1.2 mL water. After vortexing, 0.1 mL of 30% SSA was added to 0.9 mL of the cell lysate. The suspension was vortexed and allowed to stand on ice for 15 min to allow protein precipitation. Cellular protein was removed by centrifugation at 12,000 *g* for 2 min. The total GSH level in the supernatant was assessed using a modified method of Suzukake *et al.* [19]. The mixture for the assay of GSH contained 100 μL lysate, 100 μL triethanolamine buffer (1 M) pH 8.0, 700 μL NADPH and 100 μL of DTNB. The blank contained 100 μL 3% SSA instead of lysate and the control reaction contained the GSH standard in place of lysate. The mixtures were equilibrated at 30° for 3 min and the reaction started by the addition of 100 μL GSH reductase. The absorbency of 2-nitro-5-thiobenzoic acid at 412 nm was monitored on a Shimadzu model 160-A recording spectrophotometer.

Reduction of GSH levels in cell lines. In order to deduce the optimum level of BSO needed to maximise intracellular GSH reduction, cells were incubated for various time intervals at 37° in the presence of BSO concentrations from 25 μg to 100 μg/mL.

The effect of alkylating agents on GSH depleted cells. Melphalan (50 μg/mL) or chlorambucil (100 μg/mL) were added to cells (5×10^5/mL) that had previously been incubated for 20 hr at 37° in the presence of BSO (100 μg/mL). Following an additional 4 hr incubation period cytocentrifuged samples from each treatment were prepared and the percentage of apoptotic cells was calculated using morphological criteria. The cytotoxicity of melphalan and chlorambucil alone was also assessed.

Addition of chemotherapeutic drugs to GSH-depleted cells. Actinomycin-D (5 μg/mL) was added to cells (5×10^5/mL) which had previously been incubated for 20 hr with BSO (100 μg/mL). This inhibitor of GSH synthesis was maintained throughout the time the cells were exposed to actinomycin-D. At various time intervals, stained cell preparations were obtained from treatments containing actinomycin-D and BSO; percentage apoptosis occurring was calculated using morphological criteria. The cytotoxicity of actinomycin-D and BSO alone was also assessed by morphological means. Camptothecin (10 μg/mL) or etoposide (25 μg/mL) were also added to cells that had previously been incubated with BSO for 20 hr. BSO was maintained throughout exposure time to both camptothecin and etoposide. Apoptosis was assessed as for actinomycin-D treated cells.

DNA isolation and gel electrophoresis. DNA was isolated from cells which were pelleted at 200 g for 5 min. Cells were resuspended in lysis buffer (20 μL) containing 20 mM EDTA, 100 mM Tris pH 8.0, 0.8% (w/v) sodium lauryl sarcosinate and 10 μL of 1 mg/mL RNase A (prepared in 0.1 M sodium acetate, 0.3 mM EDTA pH 4.8) and incubated at 37° for 2 hr. A 10 μL aliquot of 20 mg/mL proteinase K (made up in distilled water) was added. Extracted DNA was incubated for a further 2 hr at 50°.

Electrophoresis of DNA. DNA electrophoresis was carried out in 1.5% agarose gels. A 3 μL aliquot of a 10 mg/mL ethidium bromide solution was added

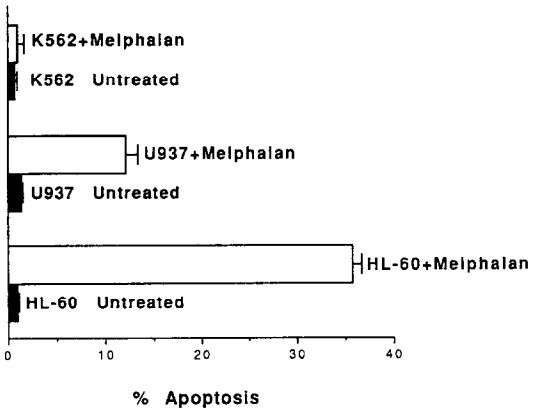

Fig. 1. Induction of apoptosis in HL-60, U937 and K562 cells using melphalan (50 μg/mL). Cells (5×10^5/mL) were incubated for a maximum of 4 hr at 37° in the presence of melphalan. The percentage apoptosis occurring in each of the samples was assessed by morphological criteria. Results are means ± SE values from three independent experiments.

Table 1. The levels of GSH present in HL-60, U937 and K562 cells

Cell line	Glutathione levels (nmol per million cells)
HL-60	8.206 ± 1.09
U937	11.048 ± 0.9
K562	14.280 ± 1.3

Cells analysed for GSH content were pelleted (200 *g* for 5 min) and GSH estimated as in Materials and Methods. Results are the means of three independent experiments. ± SE values.

to 100 mL of a 1.5% agarose solution before gel casting. Prior to electrophoresis, loading buffer (10 mM EDTA, 0.25% (w/v) bromophenol blue and 50% (w/v) glycerol) was added to each sample. Electrophoresis was carried out for 4 hr at 55 V in TBE buffer (2 mM EDTA, pH 8.0, 89 mM Tris, 89 mM boric acid). Gels were cast in apparatus supplied by CBS Scientific Co. (CA, U.S.A.) [20].

RESULTS

The effect of melphalan on HL-60, U937 and K562 cells

The alkylating agent melphalan, induced apoptosis in HL-60 cells quite readily, but did not induce significant apoptosis in U937 and K562 cell lines (Fig. 1). GSH has previously been implicated in alkylating drug resistance [11], and we investigated whether elevated GSH levels were responsible for the resistance shown by K562 and to a lesser extent U937 cells. K562 cells contained a greater amount of GSH when compared to U937 and HL-60 lines (Table 1). Since GSH levels in K562 cells were significantly higher than in the more sensitive HL-60 and U937 cells, it was reasonable to assume that the resistance in K562 cells may be related to the higher GSH levels. To determine whether there was substance to this hypothesis we reduced GSH levels and then tested the ability of melphalan to induce apoptosis. If GSH was protecting the cells from apoptosis then they ought to become more sensitive when levels were lowered.

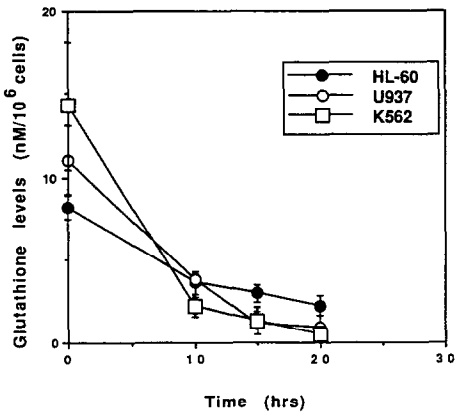

Fig. 2. Reduction of GSH levels in HL-60, U937 and K562 lines after a 20 hr incubation with BSO (100 μg/mL). Cells (5×10^5/mL) were incubated with BSO (100 μg/mL) for a range of time intervals up to a maximum of 20 hr. After each time interval the quantity of intracellular GSH present was estimated as described in Materials and Methods. Results are means ± SE values from three independent experiments.

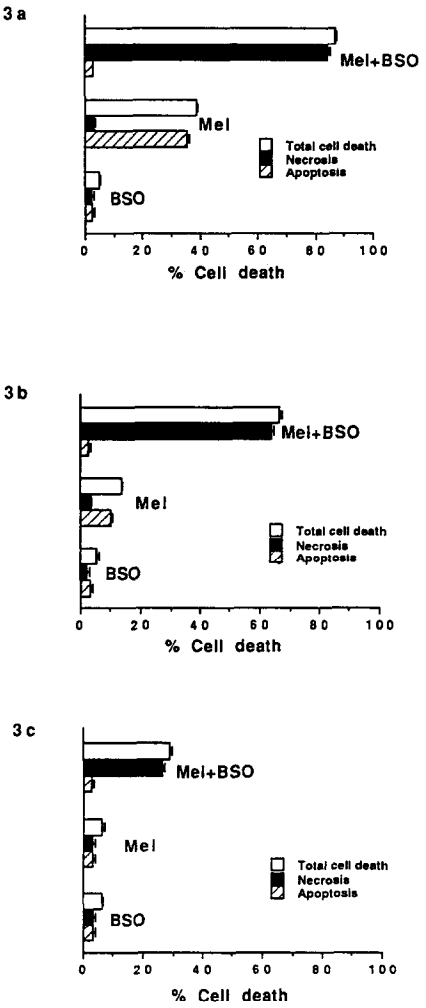

Fig. 3. The effect of reducing GSH levels upon cytotoxicity of melphalan (Mel) to (a) HL-60, (b) U937 and (c) K562 cells. After a 20 hr incubation in the presence or absence of BSO (100 μg/mL), cells were exposed to melphalan (50 μg/mL) for a further 4 hr. The percentage apoptosis and necrosis occurring in cells treated with melphalan (Mel), and cells treated with BSO and melphalan (Mel + BSO), was estimated using morphological criteria. The cytotoxicity of BSO alone was also assessed. Results are means ± SE values from three independent experiments.

Reduction of GSH levels in cell lines

Green *et al.* [11], showed that BSO, an inhibitor of GSH synthesis dramatically reduced the GSH levels in human ovarian cell lines. After a 20 hr incubation at 37° in the presence of various concentrations of BSO we found that BSO at 100 μg/mL produced the most dramatic decrease in the quantities of GSH present in each of the three cell lines (Fig. 2). We then investigated the susceptibility of K562, U937 and HL-60 cells with reduced GSH levels to melphalan-induced apoptosis. Cells which had been previously incubated in the presence of BSO (100 μg/mL, for 20 hr) were incubated for 4 hr at 37° in the presence of melphalan (50 μg/mL), BSO was maintained during incubation with melphalan. The percentage apoptosis that occurred in each treatment was assessed using morphological criteria. We found that the cytotoxicity of melphalan was enhanced in all cell types when GSH levels were low; however, under these conditions surprisingly melphalan now induced cell death via necrosis rather than apoptosis (Fig. 3a–c). This result suggested that GSH levels could affect the mode of cell death. Partially reducing GSH levels (i.e. a 5 hr pretreatment with BSO) had no effect on the toxicity of melphalan to the three cell lines used (results not shown). Alkylating agents are detoxified by GSH conjugation, principally catalysed by alpha class glutathione *S*-transferases. We have found that K562 cells have a higher level of glutathione *S*-transferase activity compared to either HL-60, or U937 lines (results not shown), thus total intracellular glutathione levels were reduced to practically zero in order to prevent different levels of drug detoxification in K562 cells compared to HL-60, or U937 cells. The characteristic banding pattern of DNA from apoptotic cells is displayed by HL-60 cells with unchanged GSH levels and which have been treated with melphalan (Fig. 4, lane 2). The random degradation of DNA from GSH depleted and melphalan treated, necrotic cells is also shown (Fig. 4, lane 3).

In order to test whether this was the case for melphalan only or for other alkylating agents as well, we investigated the effects of another alkylating agent, chlorambucil on GSH depleted cells. Like melphalan, chlorambucil induced a significant level of apoptosis in HL-60 cells within a 5 hr time period (Fig. 5); however, similar to melphalan, it failed to induce apoptosis in U937 and K562 cells within the same time span (results not shown). On lowering the GSH levels in the HL-60 line we found that the cytotoxicity of chlorambucil was significantly increased inducing cell death via necrosis (Fig. 5), a similar result was found in both U937 and K562 lines, indicating that for the alkylating agents used it seems that GSH levels influenced the resistance to, and mode of, cell death. The morphological

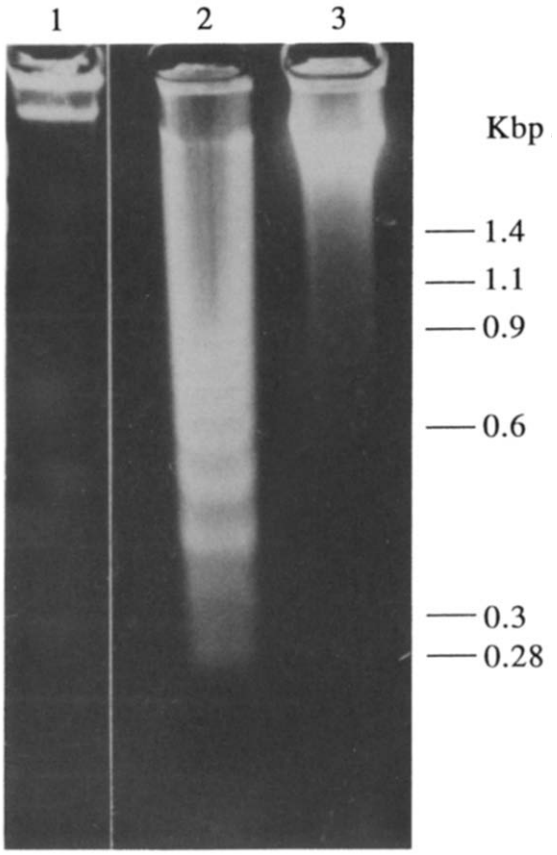

Fig. 4. Melphalan treatment (50 μg/mL) for 4 hr induces internucleosomal cleavage indicative of apoptosis in HL-60 cells (lane 2), whereas melphalan treatment of HL-60 cells previously exposed to BSO (100 μg/mL) for 20 hr induced random DNA degradation characteristic of necrosis (lane 3). DNA from untreated cells is shown in lane 1. DNA from cells exposed to melphalan, and cells exposed to BSO plus melphalan, was isolated and analysed using gel electrophoresis (see Materials and Methods).

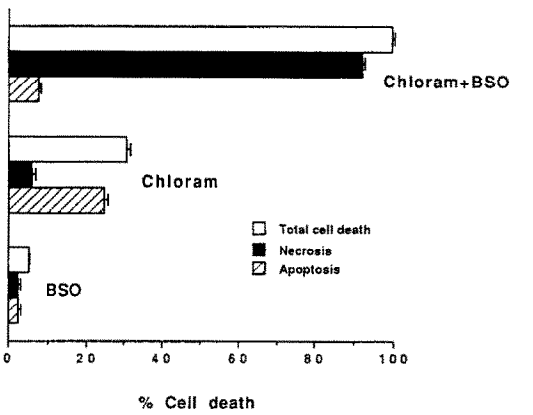

Fig. 5. The effect of BSO upon the toxicity of chlorambucil (chloram) to HL-60 cells. Cells were incubated for 20 hr with or without BSO (100 μg/mL). After incubation period the cells were treated with chlorambucil (100 μg/mL) for a further 4 hr. The percentage apoptosis, necrosis occurring was assessed in cells treated with chlorambucil and BSO (chloram + BSO), and cells treated with chlorambucil (chloram). The percentage cell death occurring in untreated cells was also assessed (result not shown). Results are means ± SE values from three independent experiments.

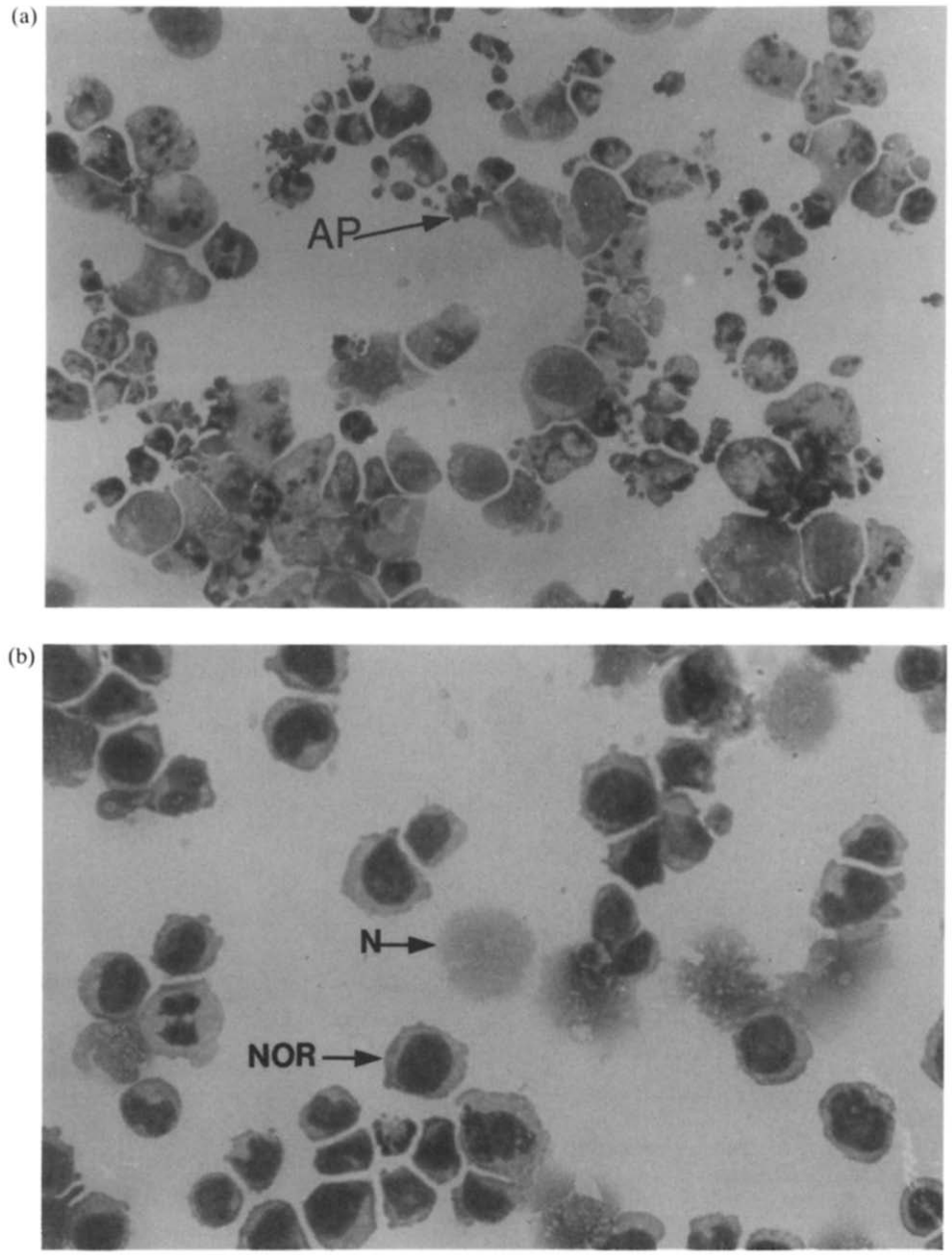

Fig. 6. (a) Morphological characteristics of apoptosis in HL-60 cells. Apoptotic cells (AP) display nuclear condensation and nuclear fragmentation typical of apoptosis in response to 4 hr incubation with melphalan (50 μg/mL). (b) Illustration of the cell swelling and plasma membrane disruption characteristic of necrosis (N). Cells with reduced GSH levels following incubation with melphalan (50 μg/mL) undergo cell death via necrosis. GSH levels were lowered by incubation of cells with BSO (100 μg/mL) for 20 hr prior to melphalan treatment. "Normal" cells (NOR) retain plasma membrane integrity and maintain a constant size.

changes occurring in apoptotic and necrotic cells are shown (Fig. 6a, b).

GSH levels and induction of apoptosis by actinomycin-D and topoisomerase inhibitors

Decreased levels of GSH steer the cell towards a necrotic mode of cell death when the inducing agent used is melphalan. To determine whether this is a general phenomenon or whether it is restricted to alkylating agents, we used two additional unrelated agents to induce apoptosis. These were actinomycin-D, an RNA synthesis inhibitor, and the topoisomerase 1 and 2 inhibitors camptothecin and etoposide. Depletion of GSH levels had no effect on the level of apoptosis induced in HL-60 cells by either actinomycin-D, etoposide or camptothecin

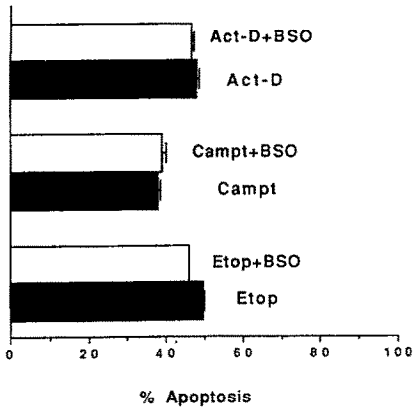

Fig. 7. The effect of reducing GSH levels upon cytotoxicity of actinomycin-D (Act-D), camptothecin (Campt) and etoposide (Etop) to HL-60 cells. After a 20 hr incubation in the presence or absence of BSO (100 μg/mL), cells were exposed to Act-D (5 μg/mL), Campt (10 μg/mL) and Etop (25 μg/mL) for a further 4 hr. The percentage apoptosis occurring in cells treated with BSO plus Act-D (Act + BSO), Campt (Campt + BSO) or Etop (Etop + BSO) and in cells exposed to Act-D, Campt or Etop was estimated using morphological criteria. Results are means ± SE values from three independent experiments.

(Fig. 7), additionally, the lowering of GSH levels in these cells had no effect on the levels of necrosis. Similar results were seen for U937 and K562 cells. These results indicate that the combination of GSH and melphalan is responsible for directing the cell towards a necrotic mode of cell death.

DISCUSSION

GSH is known to protect cells from toxic insult, presumably through conjugation with the toxin resulting in a less toxic intermediate and thus reducing the injury level to the cell. Previous studies [11, 19] have shown that GSH influences the toxicity of melphalan, but there is no information as to the mode of cell death induced. Initial experiments in this study demonstrated that melphalan induced apoptotic cell death in three haematopoietic cell types. The erythroid cell line K562 displayed a higher level of resistance to melphalan than the other two cell lines tested. This resistance may have been due to higher intracellular levels of GSH seen in K562 cells (Fig. 2).

Lowering this level by pretreating cells with BSO increases the toxicity of melphalan in all three cell lines, however the mode of cell death switches from apoptosis to necrosis. Microscopic analysis shows that HL-60 cells treated with the BSO-melphalan combination, exhibit the morphological features of necrosis (Fig. 6b). In addition, these cells also showed random DNA fragmentation (a feature of necrosis) rather than the DNA ladder pattern characteristic of apoptosis. This result was not peculiar to melphalan, since the alkylating agent chlorambucil also showed a similar effect.

We know from previously published studies that decreasing the intracellular levels of GSH increases the toxicity of certain alkylating agents [11, 19]. We also know from our work that as the concentration of apoptosis-inducing agents is increased, there is a switch from apoptosis to necrosis. A suggested explanation for this phenomenon is that at low levels of cellular insult where there is only minor damage to the cell, it still has time to activate the apoptosis programme. However, at high insult levels the injury to the cell is too severe so as not to allow a programmed mode of death and the only alternative is death by necrosis. Since pretreating cells with GSH reducing agents, artificially increases the toxicity of alkylating agents like melphalan, then it is not surprising that the mode of death switches from apoptosis to necrosis.

Support for the above contention comes from the data presented in (Fig. 7) which demonstrates that reducing GSH levels has no effect on apoptosis inducing agents which would not be expected to be affected by GSH levels.

In conclusion, lowering the levels of intracellular GSH increases the toxicity of the alkylating agents melphalan and chlorambucil, but the mode of cell death changes from apoptosis to necrosis.

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