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Misonidazole Hypoxic Cytotoxicity and Chemosensitization in Two Cell Lines with Different Intracellular Glutathione Levels

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Abstract—*Nitroimidazoles such as misonidazole (Miso) or SR-2508 are known to be cytotoxic to hypoxic cells and, with preincubation under hypoxic conditions, to sensitize cells to certain chemotherapy drugs, notably melphalan. In addition, these nitroimidazoles afford hypoxic radiosensitization; however, high intracellular glutathione (GSH) levels have been shown to significantly reduce radiosensitization by some nitroimidazoles. Using two cell lines that have an 8-fold difference in cellular GSH content, we have investigated whether inherent GSH levels influence Miso-induced hypoxic cytotoxicity, hypoxic GSH depletion, or chemosensitization to melphalan. Hypoxic incubation with varying concentrations of Miso resulted in greater cytotoxicity in Chinese hamster V79 cells than in A549 human lung adenocarcinoma cells, which have much higher GSH levels. However, the rate of GSH depletion for three concentrations of Miso was the same in the two cell lines, despite the large difference in inherent GSH levels. While the inherent sensitivity to melphalan was markedly different between the cell lines, hypoxic preincubation with 2 mM Miso with subsequent aerobic exposure to melphalan resulted in similar levels of sensitization. These results indicate that the potentiation of melphalan cytotoxicity by hypoxic Miso preincubation can occur independent of intracellular GSH levels.*

INTRODUCTION

MANIPULATION of cellular glutathione (GSH) levels [1–4] or inherent difference in cellular GSH levels [5, 6] have been shown to affect hypoxic cell radiosensitization by the nitroimidazole SR-2508. Cell lines with inherently high GSH levels were found less responsive to SR-2508 hypoxic radiosensitization. Nitroimidazoles are also known to be cytotoxic to hypoxic cells [7], and to sensitize hypoxic cells to the cytotoxicity of certain drugs, notably melphalan and the nitrosoureas [8, 9]. We have attempted to determine whether inherent differences in GSH content between cell lines would affect misonidazole (Miso) hypoxic cytotoxicity or chemosensitization to melphalan. This was done by using two cell lines that vary widely in GSH content; the Chinese hamster V79 cell line and the human lung carcinoma cell line A549.

MATERIALS AND METHODS

Stock cultures of V79 and A549 cells were maintained in exponential growth in F12 and RPMI 1640 media, respectively; both were supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Plating efficiencies were 85–95% and 50–60%, respectively.

All experiments were carried out in sealed glass flasks, gassed at 37°C with humidified 95% N_2/5% CO_2, or 95% air/5% CO_2 for oxic controls, according to the method of Ling *et al.* [10].

Briefly, sterile white rubber stoppers were placed in the necks of the flasks, and two hypodermic needles were inserted through the stopper for inlet and outlet. The flasks were then connected to the appropriate compressed gas cylinder, and gently shaken during the gassing period. Gas flow was set at 300 ml/min, and monitored with a flow meter. Previous work in our laboratory and by Dr Ling [10] has shown that radiobiological hypoxia is attained in less than 1 h of gassing with this system.

For Miso hypoxic cytotoxicity and GSH depletion experiments, the appropriate concentrations of Miso were made up in medium and added to the cells immediately prior to gassing. The flasks were gassed for 2 h (except for the 1 h points), after which time the flasks were left sealed for the remainder (if any) of the hypoxic incubation time. In the chemosensitization experiments, 2 mM Miso was added to the cells and they were gassed as described

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above. The flasks were then reaerated, rinsed, and the appropriate concentrations of melphalan were added and left on for 1 h.

After treatment, the cell monolayers were rinsed, trypsinized, counted, and plated for macroscopic colony formation. Colonies were fixed and stained after the appropriate incubation period, and colonies of 50 cells or larger were scored. For the chemosensitization experiments, enhancement ratios for the two cell lines were determined at 1% survival, and the ratio of the D_0s (slopes) of the survival curves with and without Miso was determined.

Cellular GSH levels were determined by the Tietze glutathione reductase assay [11], and protein determinations were done by the Bradford method [12]. All experiments were done a minimum of two times.

RESULTS

All three concentrations of Miso used in these experiments were more toxic to V79 than A549 cells (Fig. 1). All Miso concentrations depleted GSH from both cell lines at approximately the same rate, despite the fact that the initial GSH level in A549 cells is nearly 8-fold higher than V79 (148 ± 22 nmol/mg protein vs. 19.6 ± 5.9 nmol/mg protein). As can be seen in Fig. 2, the rate of GSH depletion increases with increasing Miso concentration, but at any given concentration, V79 and A549 show a nearly identical rate of GSH depletion.

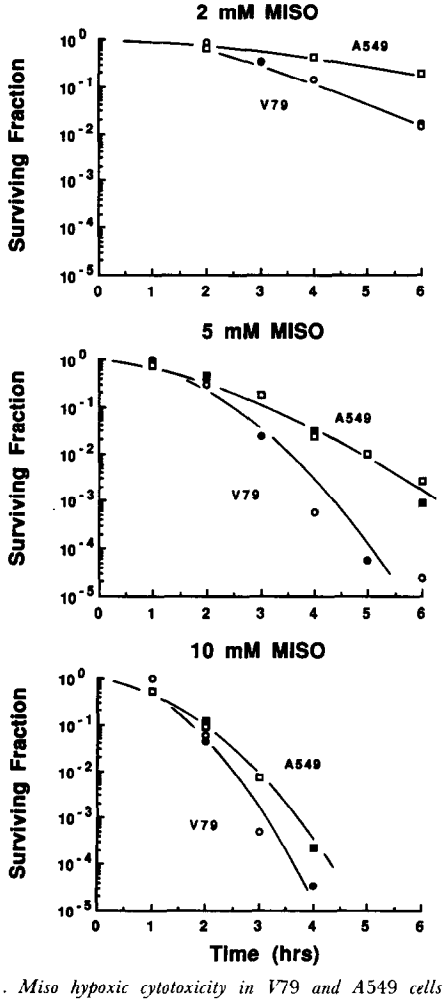

Fig. 1. Miso hypoxic cytotoxicity in V79 and A549 cells at three concentrations of Miso. Open and closed symbols are replicate experiments.

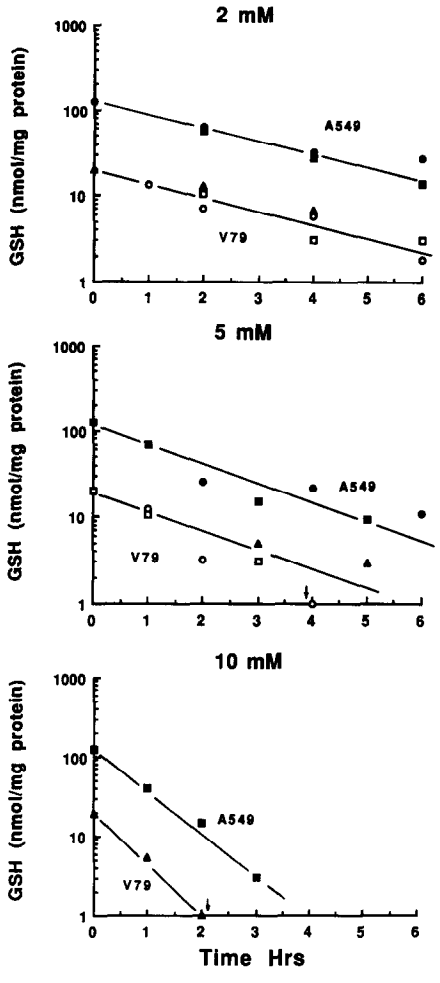

Fig. 2. Depletion of GSH by Miso under hypoxia for V79 and A549 cells. Multiple symbols represent replicate experiments.

Based on the cytotoxicity experiments, 2 mM Miso with 2 h of hypoxic incubation was chosen as the pretreatment for the chemosensitization experiments, since it produced very little toxicity in either cell line, while still providing some GSH depletion. Figure 3 shows the results of a representative experiment. It should be noted that at 10% survival, A549 cells are inherently eight times more resistant to melphalan than V79. GSH levels at the time of melphalan addition were 65 nmol/mg protein for A549 (44% of control) and 9 nmol/mg protein for V79 (46% of control). Miso pretreatment enhanced the cytotoxicity of melphalan in both cell lines. Interestingly, the extent of sensitization was approximately the same in both cell lines. The mean enhancement ratios (± S.D.) at 1% survival for four experiments were: V79, 2.9 ± 1.1; A549, 2.1 ± 0.5. Analysis by the Wilcoxon 2-sample test

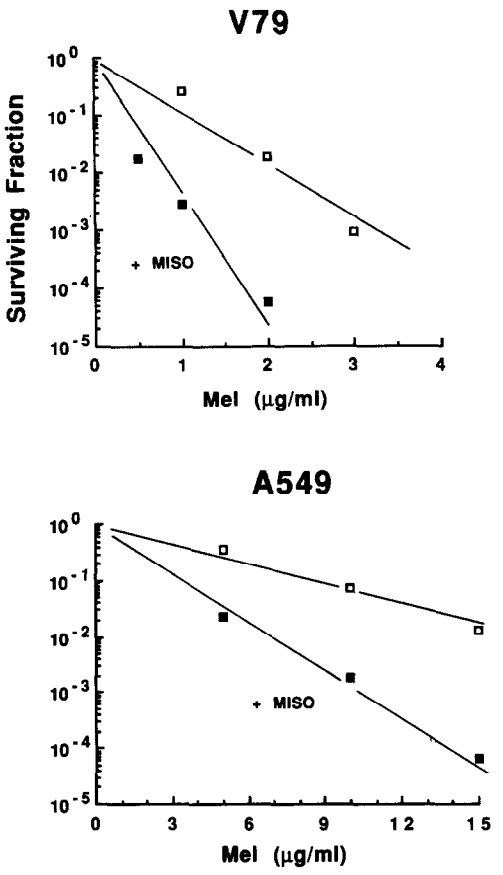

Fig. 3. Dose–response curves for V79 and A549 cells exposed to melphalan for 1 h. Open symbols represent hypoxic pretreatment only, and closed symbols hypoxic pretreatment plus 2 mM Miso. GSH levels after Miso pretreatment and prior to melphalan addition were 40 and 46% of control values for A549 and V79 cells, respectively.

indicates that the difference between these ratios is not statistically significant ($P > 0.1$). This comparison can also be made by calculating the ratio of D_0 values for the melphalan and melphalan plus Miso curves for each cell line. For the four experiments, the ratio (± S.D.) for V79 cells is 2.24 ± 1.1, and for A549 cells, the ratio is 2.36 ± 0.43.

DISCUSSION

In this study, we have shown that Miso-induced hypoxic cytotoxicity can vary considerably between cell lines that differ markedly in their GSH contents, with the cell line initially higher in GSH being less sensitive to Miso. These results are consistent with earlier work demonstrating a protective role of thiols in Miso-induced hypoxic cytotoxicity [13, 14], and agree with our earlier work [5, 6] showing that the extent of hypoxic radiosensitization by another nitroimidazole, SR-2508, is dependent on the inherent GSH level of the cell line. These data indicate GSH does play an important role in nitroimidazole radiosensitization and hypoxic cytotoxicity. The current study also shows that the rate of GSH depletion is nearly the same for V79 and A549 cells at Miso concentrations from 2 to 10 mM, despite the large difference in inherent GSH content. We have briefly studied another human lung cancer line, H460, which has a GSH content similar to that of A549, and find that the rate of GSH depletion by 2 mM Miso is similar to that seen in A549 and V79 (data not shown). The explanation for equivalent rates of GSH depletion in cell lines whose inherent GSH levels differ greatly is not clear. One possibility might be that the activities of cellular reductases are such that equivalent levels of GSH are depleted in both cell lines. Reduction of Miso under hypoxic conditions leads to the formation of reactive intermediates [15], which could then react with GSH. GSH–Miso adducts have been identified [16]; since the rate of GSH depletion is independent of the initial GSH concentration, but rather a function of the initial nitroimidazole concentration. The linear GSH depletion curves imply first order rate kinetics. The reaction rate would be dependent only on the Miso concentration, due to the large extracellular reservoir.

As can be seen in Fig. 3, at 10% survival A549 cells are 8-fold less sensitive to melphalan, and they have a nearly 8-fold higher GSH content than do V79 cells, an indication that GSH has a role in melphalan cytotoxicity. Indeed, unpublished work from our laboratory, and other studies, indicate that GSH depletion by BSO [17] or diethyl maleate [18], leads to a 1.4–1.5 fold increase in the sensitivity of CHO or V79 cells to melphalan. Melphalan-GSH adducts have been found in cells following melphalan exposure [19] and elevated levels of GSH in human tumor cell lines have been correlated with melphalan resistance [20]. Taken together, these data suggest that melphalan could react with GSH through a non-catalyzed nucleophilic reaction. At present, GSH levels in human tumor biopsies are being evaluated as a possible predictor of tumor response to agents such as melphalan [21].

The similar Miso enhancement ratios for the two cell lines following melphalan treatment are consistent with the equivalent extent of GSH depletion by hypoxic Miso preincubation. Hence, by proportional depletion of GSH in both cell lines, the extent of Miso-induced melphalan potentiation was similar. This is not to say that the only mechanism for Miso-induced chemosensitization is GSH depletion, since others have shown that for equivalent levels of thiol depletion, sensitization to melphalan was much greater when the pretreatment was 5 mM Miso vs. 5 mM buthionine sulfoximine [17]. Also, Taylor *et al.* [18] showed that thiol depletion by diethyl maleate produces a lower SER than equivalent depletion by Miso and that an increase in melphalan-induced DNA–DNA cross-links as a consequence of Miso pretreatment accounts for chemosensitization.

In summary, under hypoxia, GSH depletion by Miso occurs at the same rate in cell lines of widely varying GSH content, and the inherent GSH content plays an important role in the hypoxic cytotoxicity of Miso. However, based on the present study, melphalan potentiation by Miso preincubation is not compromised in cell lines with inherently high GSH levels.

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