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Radioprotection by glutathione ester: Transport of glutathione ester into human lymphoid cells and fibroblasts

(L-γ-glutamyl-L-cysteinylglycyl ethyl ester/γ-glutamylcysteine synthetase deficiency/glutathione synthetase deficiency/buthionine sulfoximine)

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ABSTRACT **Glutathione is not effectively transported into human lymphoid cells, normal human skin fibroblasts, and fibroblasts from patients with genetic deficiencies of γ-glutamylcysteine synthetase or glutathione synthetase. On the other hand, the monoethyl ester of glutathione, in which the carboxyl group of the glycine residue is esterified, is readily transported into these cells and is hydrolyzed intracellularly. This leads to greatly increased cellular levels of glutathione, which often exceed those found normally. Glutathione ester was found to protect human lymphoid cells of the CEM line against the lethal effects of irradiation. Under the conditions employed, complete protection was found when the ester was added prior to irradiation. Addition of the ester after irradiation was partially effective, suggesting that GSH may also function in repair processes.**

There is currently much interest in procedures that increase cellular levels of glutathione (GSH) because of growing evidence that GSH plays an important role in the protection of cells against damage by radiation and by reactive oxygen compounds and other toxic substances (1, 2). Cellular levels of GSH may be raised to some extent by increasing the supply of the amino acids required for GSH synthesis, especially cysteine, which is a substrate for the first step in GSH biosynthesis catalyzed by γ-glutamylcysteine synthetase. L-2-Oxothiazolidine-4-carboxylate, which is readily transported and converted to L-cysteine intracellularly by 5-oxoprolinase, promotes GSH synthesis (3, 4). Administration of γ-glutamylcysteine, a substrate of GSH synthetase, which catalyzes the second step, may increase cellular GSH levels to values higher than the feedback-regulated levels (5). Administered GSH does not seem to enter most cells to an appreciable extent; in contrast, there is considerable evidence that many cells export GSH (6).

An ideal cellular delivery system for GSH might be based on a derivative of GSH that is (*i*) much more effectively transported into cells than is GSH itself and (*ii*) is readily converted to GSH after transport. Observations made previously in this laboratory showed that administration of the monoethyl (or methyl) ester of GSH to mice leads to substantial increases in the GSH levels of the liver and kidney, and the findings indicated that these compounds are transported and then hydrolyzed intracellularly (7). The use of esters of GSH is attractive because (*i*) many cells appear to have high levels of esterase capable of effectively converting such compounds to GSH; (*ii*) the formation of intracellular GSH from this type of derivative does not require intracellular energy as is required for *de novo* GSH synthesis; and (*iii*) since the delivery of GSH by means of its ester does not involve GSH synthesis, the levels of cellular GSH achieved would not be subject to feedback inhibition by GSH (8).

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In the present work, we have examined the potential usefulness of the monoethyl ester of GSH as a cellular GSH delivery system in studies on several human lymphoid cell lines. The GSH ester was found to protect lymphoid cells against the effects of radiation. We have also examined normal human skin fibroblasts and fibroblasts obtained from patients with genetic deficiencies of glutathione synthetase and of γ-glutamylcysteine synthetase. The results indicate that GSH ester is very effectively transported into these cells and that high cellular levels of GSH can readily be achieved; indeed, the GSH levels are often much higher than normally found. Suspension of cells in medium containing GSH may be followed by extracellular breakdown of GSH, transport of the products, and intracellular synthesis of GSH (9). To inhibit this pathway, we pretreated the cells used in some experiments with buthionine sulfoximine, an irreversible inhibitor of γ-glutamylcysteine synthetase (10–14).

EXPERIMENTAL PROCEDURES

Materials. Human lymphoid cell lines CEM, HSB, and MOLT and skin fibroblast cultures from patients with 5-oxoprolinuria (15, 16) (GSH synthetase deficiency; GM 3878 and GM 3877) were obtained from the Institute for Medical Research, Human Cell Repository (Camden, NJ). The normal fibroblast line (CRL-1530) was obtained from the American Type Culture Collection. Cultures of skin fibroblasts from patients with γ-glutamylcysteine synthetase deficiency (17, 18) were supplied by J. Schneider (University of California, San Diego).

RPMI 1640 medium (with glutamine) (medium A) and RPMI 1640 medium (with glutamine) lacking cysteine and GSH (medium B), minimal essential medium containing Earle's salts and glutamine, MEM (with Earle's salts and glutamine, not containing GSH and cysteine), Dulbecco's phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}, Hanks' balanced salt solution lacking Ca^{2+} and Mg^{2+}, heat-inactivated fetal calf serum, penicillin, streptomycin, trypan blue, and trypsin were obtained from GIBCO. Glutathione disulfide (GSSG) reductase, NADPH, and GSH were obtained from Sigma. L-Buthionine-*SR*-sulfoximine (10–12) and GSH monoethyl ester (γ-glutamylcysteinylglycyl ethyl ester) (7) were obtained as described.

Methods. The lymphoid cell lines were grown in an atmosphere of 5% CO_2/95% air in RPMI 1640 medium containing 17% heat-inactivated fetal calf serum and 100 units each of streptomycin and penicillin per 500 ml. The cells (10^6 per ml) were used 24 hr after adding fresh medium. The cell number and viability (by trypan blue exclusion) were determined at the end of the experiments; cell viability was 90–100% at this time. For GSH determinations, 3–4 × 10^6 cells were collected by centrifugation and washed twice with phosphate-buffered saline, and the cell pellet was suspended in 0.15 ml of 0.1 M HCl and frozen. The cells were broken by freezing and

Abbreviations: GSH, glutathione; GSSG, glutathione disulfide.

thawing three times and the protein was then precipitated by adding 10% sulfosalicylic acid to a final concentration of 3.3%. After centrifugation (Beckman Microfuge), GSH analyses were performed by the GSSG reductase/dithionitrobenzoic acid recycling method (19, 20).

The fibroblasts were grown in minimal essential medium containing Earle's salts, glutamine, and 10% heat-inactivated fetal calf serum and antibiotics and were used when the cells became confluent. At the end of the experiments, trypsin was added at 25°C and, after 3–5 min, the cells were lifted from the flask surface and suspended in Hanks' solution. The cells were collected by centrifugation, washed twice with Hanks' solution, and counted. The packed cells (0.3–1 $\times 10^6$) were suspended in 0.15 ml of 0.1 M HCl and analyzed for GSH as described above.

Cells were irradiated with a ^{60}Co source (Gammacell 200; Atomic Energy of Canada, Ltd.). After irradiation, viability was monitored by trypan blue dye exclusion.

RESULTS

In the experiments described in Fig. 1, human lymphoid cells of the HSB, MOLT, and CEM lines were depleted of GSH by incubation for 24 hr in medium containing buthionine sulfoximine; the cells were then suspended in medium containing GSH ester (Fig. 1, curves 3). Within 4 hr, the intracellular levels of GSH increased substantially in each of the cell types studied, whereas there was no significant change in cellular GSH levels in untreated controls (curves 1) or in cells that were placed in medium containing an equivalent concentration of GSH (curves 2). Incubation of the cells for longer than 4 hr led to additional increases in cellular GSH, to values that were significantly higher—i.e., 2–3 nmol per 10^6 cells—than those found in these cell lines when grown under the usual conditions. In studies in which the concentration of GSH ester in the medium was 10 mM, the intracellular level increased to about 4 nmol per 10^6 cells. The usual range of cellular GSH levels found when these cells are grown under standard conditions is 0.8–1.9 nmol per 10^6 cells (or 0.8–1.9 mM).

The effect of varying the concentration of GSH ester in the medium over the range 1 to 5 mM on cellular GSH levels was determined by incubating the cells under similar conditions to those used in the studies described in Fig. 1. The cellular GSH levels achieved after 2 hr were directly related to the level of GSH ester in the medium (Fig. 2). Whereas

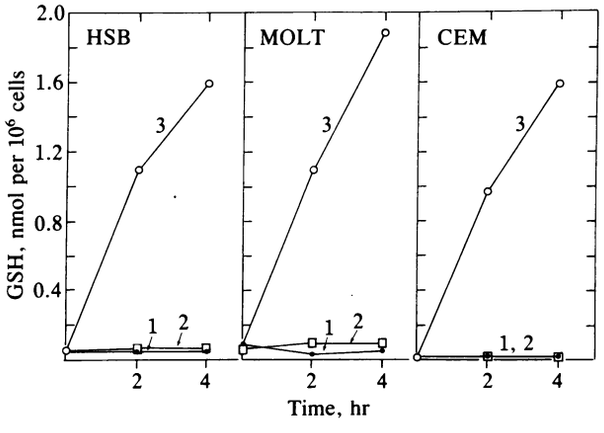

FIG. 1. Transport of GSH ester into human lymphoid cells. Cells were grown for 24 hr in RPMI 1640 medium A and then suspended in RPMI 1640 medium B containing 1 mM buthionine sulfoximine and incubated for 24 hr. The medium was removed and the cells were suspended in RPMI 1640 B medium (1.2 $\times 10^6$ cells per ml). Curves: 1, controls (no additions); 2, GSH (5 mM) was added; 3, GSH ester (5 mM) was added.

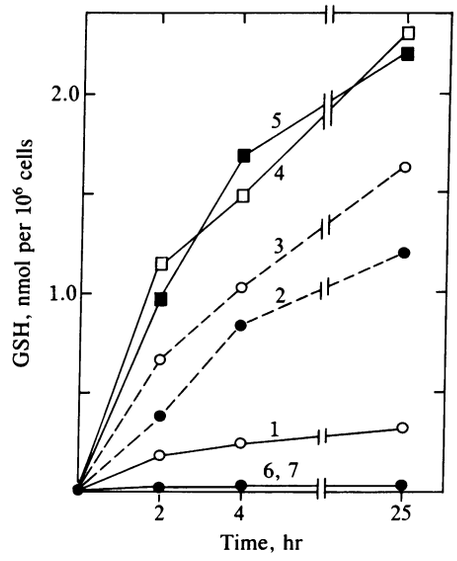

FIG. 2. Effect of concentration of GSH ester on cellular GSH levels. CEM cells were depleted of GSH as described in Fig. 1 and then suspended in RPMI 1640 medium B (1.2 $\times 10^6$ cells per ml). Curves: 1, 1 mM GSH ester; 2, 2 mM GSH ester; 3, 3 mM GSH ester; 4, 5 mM GSH ester; 5, 5 mM GSH ester plus 1 mM buthionine sulfoximine; 6, no additions; 7, GSH (5 mM) plus 1 mM buthionine sulfoximine.

buthionine sulfoximine completely inhibited formation of intracellular GSH when the cells were suspended in medium containing GSH (curve 7), buthionine sulfoximine did not affect formation of intracellular GSH when the cells were sus-

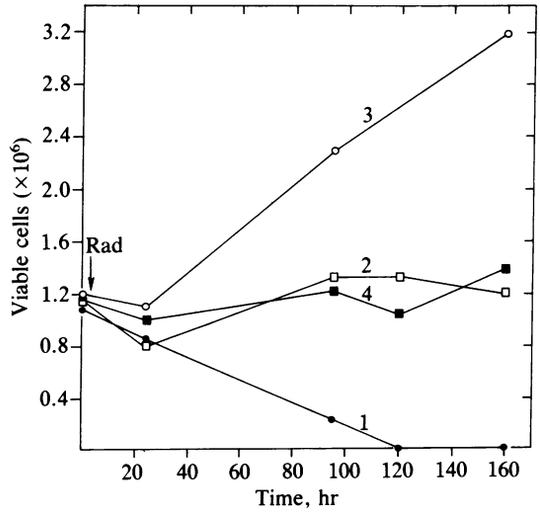

FIG. 3. Protection of lymphoid cells against radiation damage by GSH ester. CEM cells were depleted of GSH by incubation with buthionine sulfoximine (BSO) as described in Fig. 1 and then suspended in RPMI 1640 medium B containing 1 mM BSO. The following additions were then made: curves 1 and 2, none; curve 3, 5 mM GSH ester; curve 4, 5 mM GSH. At the time indicated by the arrow, (Rad; after 3 hr), the cells were irradiated with 500 rads (1 rad = 0.01 gray) and, immediately thereafter, the media were replaced in experiments 1, 3, and 4 with RPMI 1640 medium B containing 1 mM BSO and in experiment 2 with medium B containing 1 mM BSO and 5 mM GSH ester. The cells were then incubated, and the media were replaced by RPMI 1640 medium A at 24 hr and subsequently at 48-hr intervals. Curves: 1, control; 2, GSH ester was added after irradiation; 3, GSH ester was added before irradiation; 4, GSH was added before irradiation. Cell viability was initially 96%; viability at 120 hr was 0, 60, 86, and 42% for experiments 1–4, respectively.

pended in medium containing GSH ester.

The experiments described in Fig. 3 show that GSH ester protects CEM cells against the effects of irradiation. The number of viable cells decreased steadily in the controls over a period of 120 hr (curve 1). When the cells were suspended in medium containing GSH ester before irradiation (curve 3), the number of viable cells increased substantially, and indeed the number of cells increased at a rate about the same as that found for untreated cells of this cell line. Partial protection was found when the cells were suspended in medium containing GSH prior to irradiation (curve 4) and when GSH ester was added after irradiation (curve 2).

The experiments summarized in Table 1 indicate that GSH ester is also effectively transported into cultured human skin fibroblasts. These studies were carried out with normal fibroblasts, with fibroblasts obtained from two patients with a marked deficiency of glutathione synthetase, and with fibroblasts from two patients with a marked deficiency of γ-glutamylcysteine synthetase. The level of GSH usually found in normal fibroblasts is rarely higher than about 2 nmol per 10^6 cells and is generally in the range 0.8 to 1.5 nmol per 10^6 cells. After incubation with GSH ester, values of cellular GSH that were substantially higher than this were found in normal fibroblasts (Table 1, experiments 1 and 2). GSH ester was also very effectively transported into fibroblasts from patients with both types of GSH synthesis deficiency (Table 1, experiments 3–9). Suspension of the fibroblasts in medium containing GSH led to much smaller increases in cellular GSH levels than those found with GSH ester.

Table 1. Transport of GSH ester into normal fibroblasts and fibroblasts deficient in either GSH synthetase or γ-glutamylcysteine synthetase

Exp.	Cell type*	Addition	GSH, nmol/10^6 cells
1	Normal	None	0.8–1.5
		GSH ester	6.5
2	Normal†	None	<0.1
		GSH	0.14
		GSH ester	3.6–5.2
3	GSH synthetase (3878)	None	0.6
		GSH ester	6.3–9.0
4	GSH synthetase† (3878)	None	0.2
		GSH	0.4
		GSH ester	8.4
5	GSH synthetase (3877)	None	0.4
		GSH ester	4.8
		GSH ester (3 mM)	1.6
		GSH ester (10 mM)	7.9
6	GSH synthetase† (3877)	None	0.15
		GSH	0.5
		GSH ester	5.0
7	γ-Glu-Cys synthetase (PC)	None	0.18
		GSH ester	7.4
8	γ-Glu-Cys synthetase (HK)	None	0.55
		GSH	1.3
		GSH ester	17.5
9	γ-Glu-Cys-synthetase† (PC)	None	0.08
		GSH	0.23
		GSH ester	7.4

Flasks containing confluent attached cells were used. The medium was replaced with minimal essential medium (with Earle's salts and glutamine; without GSH and cysteine) containing, as indicated, 5 mM GSH ester or 5 mM GSH. After incubation in 5% CO_2/95% air for 4 hr, the cells were separated, washed, and analyzed for GSH. Cell viability was 88–95% at the end of the experiments.
*Normal or enzyme deficient.
†Cells were treated with 1 mM buthionine sulfoximine in α-minimal essential medium for 24 hr prior to the experiment.

DISCUSSION

These studies indicate that GSH monoethyl ester has properties that are suitable for use in a cellular delivery system for GSH. In the course of this work, we have found that certain other mono- and diesters of GSH and the corresponding amides are also transported into cells and converted to GSH intracellularly. We have thus far preferred to use GSH monoethyl ester because the other intracellular cleavage product, ethanol, is less likely to be seriously toxic than methanol, higher alcohols, and ammonia. GSH diesters were found to be toxic in mice. Our findings suggest that esters of the glycine carboxyl group of GSH are cleaved more readily by esterase than those of the glutamate carboxyl moiety.

Although it would be expected that GSH ethyl ester would be readily susceptible to oxidation and to hydrolysis in tissue culture media and *in vivo*, the present and previous (7) results indicate that GSH ethyl ester has sufficient stability *in vitro* and *in vivo* to serve as a substantial substrate for transport. Analysis of the suspending medium during the course of these experiments shows that appreciable oxidation of GSH monoethyl ester occurs as well as some cleavage of the ester group. Because both oxidation and hydrolysis of the ester occur, together with some formation of GSSG, it has thus far been difficult to quantitate each type of breakdown of GSH ester in the medium. Such breakdown is markedly affected by various components of the culture medium (e.g., metal ions, serum proteins) and also by pH. In this connection, it is important to maintain the pH of solutions of the ester at values less than 7.6; even brief periods at higher pH values may lead to rapid hydrolysis.

A number of studies have indicated that the transport of GSH monoethyl ester is not significantly affected by the simultaneous presence of relatively high concentrations of GSH. This is consistent with previous findings that GSH is not appreciably transported (9, 13) and with the interpretation that GSH ester is much more readily accepted into the cell membrane. Transport of the ester seems to be proportional to concentration of the ester in the medium over a wide range; this process probably occurs by diffusion. Further studies on the properties of this transport phenomenon are needed.

The protection of lymphoid cells observed in the presence of GSH ester is in accord with earlier findings (9), which showed that protection requires intracellular GSH. That partial protection was found in cells that were incubated with GSH prior to irradiation probably reflects some uptake of GSH degradation products and intracellular GSH synthesis, as previously observed (9). That partial protection was found when GSH ester was added after irradiation suggests that some cellular damage is reversible and that GSH may function in repair processes as well as in protection.

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