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INCREASE BY VINBLASTINE OF OXIDIZED GLUTATHIONE IN CULTURED MAMMALIAN CELLS

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Abstract—When added to monolayer cultures of H-35 rat hepatoma cells, vinblastine sulfate caused a marked increase in cellular levels of oxidized glutathione, going from a zero-time value ranging from 0–24 pmoles/mg protein to 1736 pmoles/mg protein in 4 hr. During this time, there was little measurable change in cellular levels of reduced glutathione. This effect of vinblastine, which was concentration-related, was produced by other vinca alkaloids, vincristine and vindesine, but not by other classes of anticancer drugs, such as those represented by adriamycin and methotrexate. Vinblastine inhibited [^{3}H]leucine incorporation into protein in this system, and this effect was also concentration-related. A reciprocal relationship existed between the cellular levels of oxidized glutathione and the inhibition of protein synthesis produced by vinblastine. The vinca alkaloids may alter cellular glutathione levels and, consequently, inhibit protein synthesis, possibly through their effects on microtubule dynamics.

Recent studies on the physiological regulation of tubulin/microtubule dynamics have generated the plausible hypothesis that these processes are mediated by glutathione, which may be involved in the maintenance of the sulfhydryl groups of tubulin in a reduced form for assembly [1–4]. Tubulin apparently cannot be polymerized if the sulfhydryl groups are oxidized or if oxidized glutathione (GSSG) is present [1, 5, 6]. Moreover, it appears that mixed disulfides of protein and glutathione may act as a physiologic reservoir for glutathione [4].

It is known that the vinca alkaloids bind specifically to tubulin molecules and thus prevent the assembly and cause the disassembly of microtubules [7–9]. The actual mechanism by which these drugs perturb microtubule dynamics, however, remains obscure. Since glutathione may be involved normally in the assembly and disassembly of microtubules, it is appropriate to ask whether the vinca alkaloids also alter cellular glutathione levels and, if so, whether this can account for some of the other actions of these drugs. Evidence is presented to show that vinca alkaloids produce a marked increase in the cellular levels of GSSG, and that this phenomenon has consequences for the synthesis of proteins.

MATERIALS AND METHODS

Chemicals and supplies. The following chemicals and supplies were purchased: reduced glutathione (GSH), GSSG and diazenedicarboxylic acid bis (*N,N*-dimethylamide) (diamide), from Calbiochem (San Diego, CA); 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide (NEM), sodium dodecylsulfate (SDS) and bovine serum albumin (fraction V) from the Sigma Chemical Co (St. Louis, MO); [4,5-^{3}H]-L-leucine, 6 Ci/mmole, from Schwarz–Mann (Orangeburg, NY); cell culture media and trypsin from Gibco (Grand Island, NY); fetal calf serum from Flow Laboratories (Rockville, MD); plastic culture dishes from the Lux Scientific Corp. (Newbury Park, CA); and plastic culture flasks from Falcon (Oxnard, CA). All other chemicals and supplies were also obtained from commercial sources. Vinca alkaloids were supplied by Eli Lilly & Co. (Indianapolis, IN), through the courtesy of Dr. Robert J. Hosley. Adriamycin was obtained from Adria Laboratories (Wilmington, DE), and methotrexate from the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD).

Cells and culture conditions. Reuber H-35 rat hepatoma cells (obtained from Dr. F. Kenney, through Prof. E. S. Canellakis) were grown in a humidified chamber at 37° in an atmosphere of 95% air and 5% CO_2 in Eagle's Minimal Essential Medium (Hanks' salts), supplemented with a 4-fold higher level of amino acids, vitamins and sodium pyruvate, as well as 13% heat-inactivated fetal calf serum.

Stock cells were grown in 75-cm^2 flasks, collected by mild trypsinization, and resuspended for replicate plating in dishes (60 × 15 mm) at the appropriate cell density in the medium described. After 3 days of growth (cell density ~ 2–4 × 10^6/dish), the medium was removed, the cell layer was washed (once) with 2.5 ml of warm 0.9% NaCl, and serum-free, unsupplemented Eagle's Minimal Essential Medium was added to each dish. It was determined that, under these conditions, cells remain viable for at least 6 days and will resume normal growth and metabolic activities after the readdition of the serum-containing, supplemented medium.

Assays for GSH, GSSG and protein. Cellular GSH was measured by a modification of the method of Beutler *et al.* [10]. Fresh, serum-containing medium (± vinblastine, VLB) was aspirated from the dishes at various times, and the monolayers were washed rapidly (twice) with 2.5 ml of 0.9% NaCl. Precipi-

tating solution (0.8 ml, containing 30% NaCl, 0.2% Na_2EDTA, and 1.67% glacial meta-phosphoric acid) was added to each dish and after 10 min the contents were scraped, removed to a tube, and centrifuged at 10,000 *g* for 10 min. To 200 μl of the supernatant fluid was added 800 μl of Ellman reagent (700 μl of 0.3 Na_2HPO_4 and 100 μl of 0.4 mg/ml of DTNB in 1% sodium citrate). The optical density was read at 412 nm against a blank of precipitating solution only, and the content of GSH was calculated by using a value of 13,600 M^{-1} for the molar extinction coefficient of the DTNB–GSH complex [10].

Cellular GSSG was measured by a modification of the method of Burchill *et al.* [4]. Fresh, serum-containing medium (± VLB) was aspirated from the dishes at various times and the monolayers were washed (once) with 2.5 ml of ice-cold 0.9% NaCl. One milliliter of 0.1 M NEM was added to the cells, which were then scraped, removed to tubes and centrifuged at 10,000 *g* for 10 min. The supernatant fluid was decanted and the pellet was treated with 500 μl of cold 10% trichloroacetic acid (TCA), mixed well, and centrifuged again. This second supernatant fluid was added to the first and extracted five times with ether; residual ether was removed by bubbling N_2 through these supernatant fractions. The samples were then assayed for GSSG by the Tietze method, as described by Burchill *et al.* [4]. Protein was determined by the method of Lowry *et al.* [11].

Protein synthesis was estimated by measuring the incorporation of [^{3}H]leucine into TCA-precipitable material. Three-day, serum-starved monolayers were re-fed with fresh medium containing serum and [^{3}H]leucine (1 μCi/ml). In other experiments, cultures were pulsed with [^{3}H]leucine (3 μCi/dish) 5 min before collecting the cells. At the times indicated, the medium was aspirated, the dishes were washed three times with 2.5 ml of ice-cold 0.9% NaCl, and the monolayers were dissolved in 2.0 ml of 0.1% SDS. Cold 20% TCA (500 μl) was added to 500 μl of the SDS-digest, chilled, and collected on Millipore filters which were counted for radioactivity. Protein concentration in these experiments was determined by the method of Bradford [12].

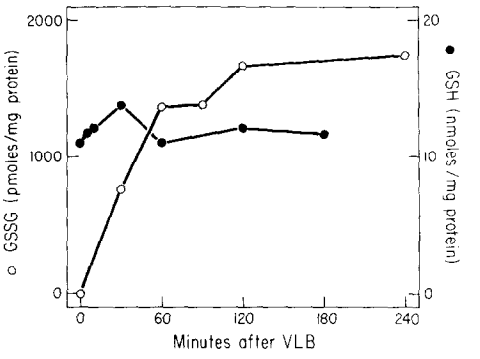

Fig. 1. Effect of VLB (8.2×10^{-8} M) on GSSG and GSH levels in monolayer cultures of H-35 rat hepatoma cells. Three-day, serum-starved monolayers were re-fed with fresh medium ± VLB; at the times indicated, cells were harvested and analyzed for glutathione content as described in the text. Key: (○), GSSG, in pmoles/mg protein; (●) GSH, in nmoles/mg protein.

RESULTS

Effects of vinca alkaloids on cellular levels of GSH and GSSG. The effects of VLB on GSH and GSSG were examined in serum-starved monolayer cultures of H-35 rat hepatoma cells that had been re-fed with fresh serum-containing medium. Under these conditions, cells that had been growth arrested resumed normal growth; the vinca alkaloids interfered with this. It can be seen in Fig. 1 that 8.2×10^{-8} M VLB, which can inhibit 2-day cell growth by 50–66 per cent,* had little effect on the level of cellular GSH during a 3-hr period of study. The drug caused a great increase in the cellular content of GSSG, however, which in this experiment rose from a zero-time value of 0 pmoles/mg protein (undetectable) to a 4-hr value of 1736 pmoles/mg protein; over this time period, GSSG levels in untreated controls changed very little (0–23.7 pmoles/mg protein). Since the ratio of GSH to GSSG in the cell is normally ~ 1000 : 1, such large changes in cellular GSSG could be mediated by slight, barely detectable changes in GSH.

Dose–response experiments revealed that this effect of VLB on cellular GSSG levels was concentration dependent. Figure 2 shows that, by increasing the concentrations of VLB in the medium, the cellular GSSG content rose to correspondingly higher levels, and this occurred earlier after drug addition. These effects on cellular GSSG also occurred with the other vinca alkaloids (vincristine and vindesine), but not with the other oncolytic drugs (methotrexate, adriamycin) (Table 1). Additionally, the vinca alkaloids varied in their abilities to produce this effect (vinblastine > vincristine > vindesine). Preliminary experiments indicated that this effect of VLB on GSSG levels could be reversed within 30–60 min after washing the dishes and incubating the cells in drug-free medium.*

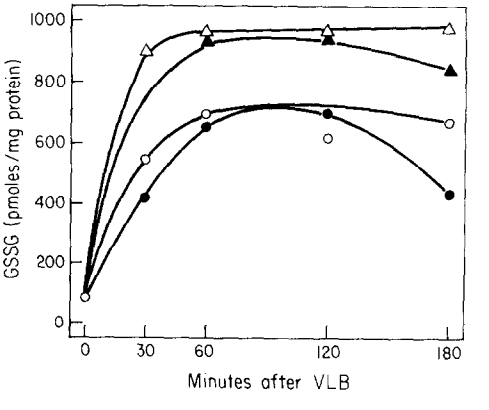

Fig. 2. Effect of VLB on GSSG levels in H-35 monolayers. See legend to Fig. 1 for details. Key: concentrations of added VLB ($M \times 10^{-8}$): (●) 5.5; (○) 8.2; (▲) 11.0; and (△) 22.0.

* W. T. Beck, unpublished observation.

Table 1. Cellular GSSG levels in H-35 monolayer cultures 3 hr after addition of drugs*

Expt.	Drug	Concentration (M)	GSSG (pmoles/mg protein)
I	None	Serum-starved	28.3
		Re-fed cultures	21.7
	Vinblastine	5.5×10^{-8}	436
		8.2×10^{-8}	664
		1.1×10^{-7}	838
		2.2×10^{-7}	968
	Vincristine	1.1×10^{-7}	282
	Adriamycin	1.8×10^{-7}	49.0
II	None	Serum-starved	0†
		Re-fed cultures	0
	Vinblastine	1.1×10^{-7}	480
	Vindesine	5.5×10^{-8}	45.3
		1.1×10^{-7}	74.8
		2.2×10^{-7}	159
	Methotrexate	1.0×10^{-6}	0
		1.0×10^{-5}	0
		1.0×10^{-4}	0

* Three-day, serum-starved monolayer cultures were re-fed with fresh serum-containing medium ± drugs at the designated concentrations. Cells were prepared and the levels of GSSG were measured according to the methods described. Each measurement, the average of five dishes that were pooled, was done in duplicate.

† Value for the sample was below that for the blank.

Effects of VLB on protein synthesis. It has been demonstrated that GSSG inhibits protein synthesis in eukaryotic and other systems [13–15]. Although the mechanism of this inhibition is not clear, it may be related to an inability of ribosomal proteins to be maintained in the reduced state or, more specifically, to the activation of an inhibitory protein kinase [15]. It was shown some years ago that the vinca alkaloids inhibit protein synthesis in murine cells, but the mechanism of this effect has never been resolved [16–18]. In light of these observations, we determined if inhibition of protein synthesis was a consequence of the elevated levels of GSSG produced by vinca alkaloids. Figure 3 illustrates that VLB inhibited protein synthesis in a dose-related manner, becoming progressively greater with time, so that by 180 min, 8.2×10^{-8} M VLB produced an inhibition of 25 per cent in the incorporation of [^{3}H]leucine into TCA-precipitable material, and 22.0×10^{-8} M VLB inhibited [^{3}H]leucine incorporation by 60 per cent. Under these conditions, VLB had no noticeable effect on [^{3}H]leucine uptake, when compared with controls (data not shown).

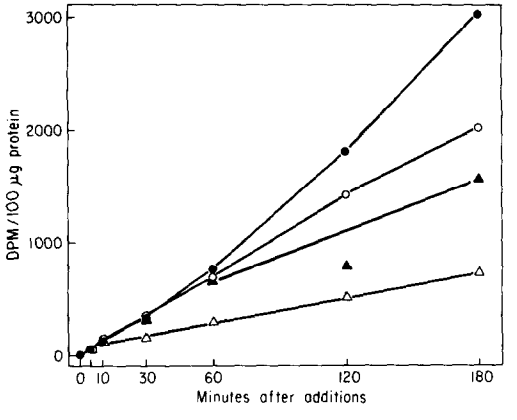

Fig. 3. Effect of VLB on [^{3}H]leucine incorporation into TCA-precipitable material in monolayer cultures of H-35 cells. Three-day, serum-starved monolayers were re-fed with fresh medium ± VLB and containing [^{3}H]leucine. At the times indicated, the monolayers were harvested and analyzed for radioactivity and protein as described in the text. Each point is the mean of triplicate determinations; the standard deviation for each point was ± 5–10 per cent of the mean. Key: (●) control, re-fed, drug-free cultures; VLB-treated dishes: (○) 8.2×10^{-8} M; (▲) 11.0×10^{-8} M; (△) 22.0×10^{-8} M.

Relationship between cellular levels of GSSG and protein synthesis. When inhibition of protein synthesis was expressed as the ratio of drug-treated to control leucine incorporation, and then plotted in relation to VLB-induced changes in the concentration of cellular GSSG with respect to time, the results shown in Fig. 4 were obtained. As the concentration of VLB was increased, the highest level of GSSG attained was increased, and this maximum was reached earlier. Concomitantly, progressive inhibition of protein synthesis occurred as the concentration of VLB was raised—this effect also occurred earlier with higher VLB concentrations. Figure 4 illustrates a reciprocal relation between the level of GSSG and the inhibition of protein synthesis produced by increasing concentrations of VLB. Thus, the previously reported inhibition of protein synthesis produced by vinca alkaloids may be mediated through the effects of these drugs on the level of GSSG within cells.

The results in Fig. 4, however, do not prove that elevated levels of GSSG are responsible for the observed inhibition of protein synthesis. To study this relationship, experiments were done with diam-

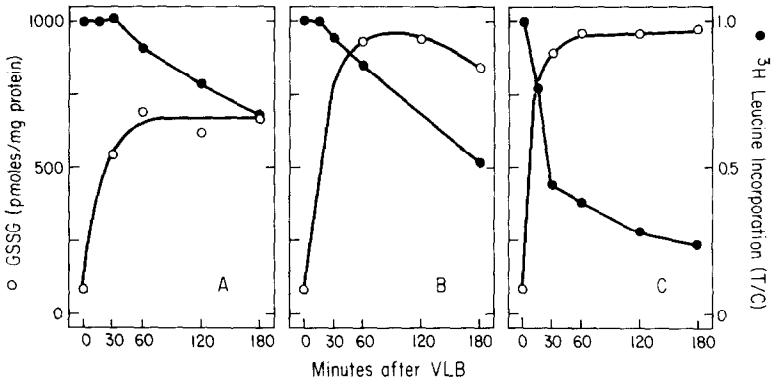

Fig. 4. Reciprocal relation between oxidized glutathione (GSSG) and protein synthesis ([^{3}H]leucine incorporation) produced by VLB. Serum-starved monolayer cultures of H-35 cells were re-fed with medium containing [^{3}H]leucine and VLB at 8.2×10^{-8} M (A), 11.0×10^{-8} M (B), or 22.0×10^{-8} M (C). See legends to Figs. 2 and 3 for other details. Key: (○) GSSG, in pmoles/mg protein; and (●) [^{3}H]leucine incorporation, VLB-treated/control.

ide (diazenedicarboxylic acid bis[*N,N*-dimethylamide]) a glutathione-oxidant [19]. This compound, as do the vinca alkaloids, blocks the polymerization of tubulin into microtubules and promotes the disassembly of microtubules [1]. These effects on microtubule dynamics are due apparently to oxidation of GSH (and protein-SH) to GSSG (and protein-SSG). We reasoned that, if this compound causes a transient elevation in cellular levels of GSSG, it should be able to block protein synthesis over a similar time period. Figure 5 shows that diamide produced a rapid increase in cellular GSSG that reached a peak at 2.5 min, followed by a decline to control levels at 60 min. Accompanying the increase in GSSG, there was a decrease in protein synthesis that reached a nadir at 10 min and returned to normal at 60 min. Although these results with diamide are not conclusive, they are suggestive and lend support to our VLB data. It appears, therefore, that an inverse relationship exists between elevated GSSG levels and subsequent inhibition of protein synthesis.

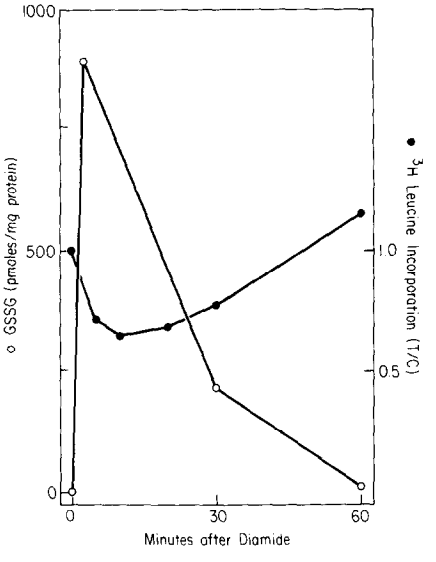

Fig. 5. Effects of diamide on the level of GSSG and the rate of [^{3}H]leucine incorporation into TCA-precipitable material in monolayer cultures of H-35 cells. Three-day, serum-starved monolayers were re-fed with fresh medium ± diamide (final concentration, 1×10^{-4} M). Five minutes before harvesting the cells, the monolayers were pulsed with 3 μCi [^{3}H]leucine per dish. At the times indicated, the monolayers were harvested and analyzed for GSSG, radioactivity, and protein as described in the text. Each point is the mean value from three dishes. Key: (○) GSSG, in pmoles/mg protein; and (●) [^{3}H]leucine incorporated, diamide-treated/control (T/C).

DISCUSSION

These studies show that vinca alkaloids produce a marked elevation in the level of cellular GSSG and that, as a consequence, the synthesis of proteins is inhibited. How might these agents cause a rise in GSSG? It is possible to envisage a state of dynamic equilibrium of microtubules ⇌ reduced tubulin ⇌ oxidized tubulin, as depicted in Fig. 6. Vinca alkaloids bind to tubulin molecules, thereby preventing their polymerization into microtubules and causing the depolymerization of these microtubules [7, 8]. Since the regulation of the assembly and disassembly of microtubules is dependent on free -SH groups [5, 6], and because glutathione appears to be a cellular compound necessary for the maintenance of the -SH groups of tubulin and other proteins in the reduced form [1–4], it is conceivable that, by binding to tubulin subunits and interfering

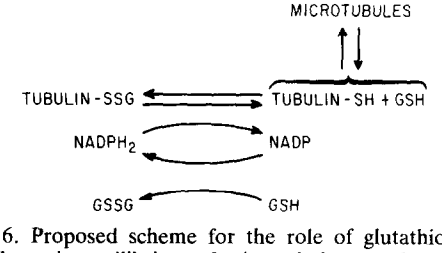

Fig. 6. Proposed scheme for the role of glutathione in the dynamic equilibrium of microtubules ⇌ reduced tubulin ⇌ oxidized tubulin.

with microtubule dynamics, the vinca alkaloids perturb the equilibrium between tubulin and microtubules and between GSH and GSSG. As a consequence, the cell would utilize its GSH-reducing capacity in an attempt to maintain the activity of the assembly–disassembly system. Since the ratio of GSH to GSSG in the cell is ~ 1000:1, the cell would be much more sensitive to changes in the GSSG levels than to changes in the GSH levels; very small changes in cellular GSH would be reflected in large changes in the levels of GSSG, as shown in this paper. It is also possible that the vinca alkaloids increase the levels of GSSG by inhibiting glutathione reductase or by interfering with the production of reducing equivalents ($NADPH_2$) through some as yet undescribed effect on the activity of the pentose shunt pathway. All of these possibilities can be tested experimentally.

In conclusion, VLB causes an increase in the cellular level of GSSG, which in turn may be the cause of the inhibition of protein synthesis observed. Since cell death might result from the prevention of the synthesis (or the degradation) of a few key proteins, this effect of VLB on cellular levels of GSSG may be a critical event leading to the lethal effect of these drugs on cells.

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