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Serial determinations of glutathione levels and glutathione-related enzyme activities in human tumor cells *in vitro*

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Recent studies have examined the role of intracellular glutathione (GSH) and GSH-related enzymes in the development *in vitro* of resistance to antineoplastic drugs by tumor cells [1]. GSH levels 2- to 4-fold higher than that of the parent line have been described in human ovarian cancer cells selected for resistance [2, 3]. This has also been documented in murine leukemia cells resistant to melphalan and mitomycin C [4, 5]. Glutathione-*S*-transferase (GST) was found to be elevated 2-fold compared to controls in murine mammary carcinoma cells selected for resistance to alkylating agents [6], and at least 50-fold in human breast cancer cells with pleiotropic drug resistance after selection for Adriamycin resistance [7]. Levels of both GSH and GST were found to be 4- to 6-fold higher in a human ovarian cancer cell line established from a patient clinically resistant to combination chemotherapy including cisplatin compared to another line established prior to therapy [8]. The redox cycling of GSH by GSH-peroxidase (GSH-Px) and GSH-reductase (GSH-R) has been shown to play an important role in cellular defense against peroxide damage in cultured cells, although there have been few studies comparing the activity of either enzyme among various cell lines [8–10].

Tissue culture conditions have been shown to influence significantly the cellular GSH level in a human lung cancer cell line [11]. In particular, serum concentrations and time from last passage predictably affect GSH levels. To determine if growth-related fluctuations may conceal or exaggerate differences between cell lines, we have serially determined the levels of GSH and three GSH-dependent enzymes in a human ovarian cancer cell line derived from an untreated patient (A2780) and in a subline selected for resistance to cisplatin (2780^{CP}).

Methods

Cell lines and tissue culture. The A2780 human ovarian cancer cell line, established from an untreated patient, was furnished by Dr. S. Aaronson of the National Cancer Institute. The cells used in this study were passage 32 of this cell line. Subline 2780^{CP}, approximately 7-fold resistant to cisplatin compared to the A2780 line, was obtained by exposure of the A2780 cell line to cisplatin as the concentration was gradually increased to 8 μM [12]. The cells studied here were passage 116 of this cell line and had been drug-free for greater than 6 months. The cell lines were plated at a density of 1.5×10^6 cells/75 cm^2 tissue culture flask and were grown as monolayers in drug-free RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 0.25 units/ml insulin, 2 mM glutamine, and antibiotics (Grand Island Biological Co., Grand Island, NY). Medium was changed on days 1, 4 and 7 after plating.

GSH assays. Cell suspensions from multiple monolayer cultures of each line were obtained by trypsinization of cells at the specified times. Cell numbers were determined by a Coulter counter (model ZBI, Coulter Electronics, Hialeah, FL). The total glutathione content, reduced (GSH) plus oxidized (GSSG), was assayed in an aliquot of each suspension using a modification of the method of Suzukake *et al.* [13] in which the rate (rather than the total extent) of 2-nitro-5-thiobenzoic acid formation is measured spectrophotometrically. The initial velocity of this reaction is linearly related to the total GSH content over sample concentrations of 1–25 μg/ml.

Enzyme assays. Cell homogenates were prepared by hypotonic lysis followed by disruption in a Potter–Elvehjem homogenizer. All assays were run in triplicate for each of three homogenates per cell line and time point. GSH-Px was measured according to Paglia and Valentine [14] with H_2O_2 as substrate; GSH-R was assayed as described by Horn [15]. For both, activity is expressed as nmole NADPH oxidized/minute. GST was estimated using the method of Habig and Jakoby [16] with chlorodinitrobenzene as substrate. Protein was determined by the method of Lowry *et al.* [17] using fatty-acid-free bovine serum albumin as standard.

Statistical analysis. For each function measured, the pattern of change over time between the two cell lines was compared statistically using the repeated measures analysis described by Winer [18]. Rather than comparing the cell lines at each time point using a simple two-sample *t*-test, this method takes into consideration the fact that measurements are taken repeatedly on the same cells. Ignoring this data structure may lead to incorrect interpretation.

Results and discussion

The doubling time of the cell lines was 22–25 hr, and both cell lines at 2, 4, 6, and 9 days after plating were in approximately early, mid, late logarithmic and early stationary phases of growth respectively.

Serial GSH determinations for the two cell lines are shown in Table 1. In accord with Post *et al.* [11], total GSH levels were maximal in both lines early after subculture (day 2) and then declined. Fluctuation in GSH level over time was greater in the 2780^{CP} cell line, which varied from 6.1 to 24.2 nmoles/10^6 cells (4-fold), whereas in the A2780 line GSH varied from 2.4 to 5.9 nmoles/10^6 cells (2.4-fold). Total GSH levels were significantly higher ($P < 0.05$) at all times in 2780^{CP}, however, the ratio of the mean GSH levels in the two cell lines ranged from 1.8 on day 6 to 4.1 on day 2. Were the samples measured only on day 6, the difference might easily have been discounted. Furthermore if day-4 A2780 cells had been compared to day-6 resistant cells, the difference would have appeared even smaller (1.3-fold). The pattern of change over time in absolute GSH level also differed between the two cell lines. The largest difference between the cell lines was on day 2 when GSH levels were highest in both. The subsequent decline in GSH levels was dramatic in the 2780^{CP} cell line and more gradual in the

Table 1. total cellular glutathione levels at each time point in the two cell lines

Day	GSH + GSSG (nmoles/10^6 cells) A2780	2780^{CP}
2	5.9 ± 0.12	24.2 ± 1.20
4	4.5 ± 0.37	10.7 ± 0.30
6	3.5 ± 0.32	6.1 ± 0.37
9	2.4 ± 0.02	7.5 ± 0.37

Relative cell volumes are 1.00 and 1.08 for the A2780 and 2780^{CP} lines, respectively, measured on a Coulter Counter equipped with a Channelizer. Values are means ± S.E.M., N = 3.

Table 2 Glutathione-dependent enzymes in human ovarian cancer cells measured over time since passage

Days after passage	GSH-peroxidase		GSH-reductase		GSH-*S*-transferase	
	A2780	2780^{CP}	A2780	2780^{CP}	A2780	2780^{CP}
2	8 0 ± 0 81	27 4 ± 1 96	26 8 ± 1 39	60 6 ± 7 06	83-1 ± 12 09	57 7 ± 4 69
4	14 8 ± 1 59	14 0 ± 1 03	38 7 ± 2 27	36 4 ± 3 34	118 1 ± 8 64	92 6 ± 8 38
6	8 3 ± 1 30	9 9 ± 1 36	34 7 ± 1 77	44 7 ± 4 73	73 1 ± 4 48	89 3 ± 3 15
9	9 0 ± 1 36	13 1 ± 0 73	39 3 ± 5 19	33 6 ± 3 39	79 6 ± 6 09	70 2 ± 1 28

Values are mean units/mg protein ± S E M , N = 3 GSH-peroxidase and GSH-reductase 1 unit = 1 nmole NADPH oxidized/min, GSH-*S*-transferase 1 unit = 1 nmole increase in 340 nm absorbance/min

A2780 line The significance of this difference between the cell lines in the early fluctuation rate of GSH is not known but may reflect generally enhanced resistant-cell response to trypsinization As shown below, changes in the activities of both GSH-Px and GSH-R, which form a GSH redox cycle, paralleled the changes in GSH levels in the cisplatin-resistant cells

Table 2 shows the results of serial enzyme assays in these two cell lines at different points in time All three of the enzymes measured also changed with time during growth in culture, by as much as 2-fold ($P < 0\ 01$ for each enzyme) The pattern of fluctuation differed significantly between the two cell lines for GSH-Px and GSH-R ($P < 0\ 01$), while that of GST was not different in the two cell lines In the A2780 cells, the enzymes increased in activity after passage and reached a peak on day 4, in mid-logarithmic growth Their activities return to baseline or remained essentially stable thereafter In the 2780^{CP} cells, the GST activity was lower than that of the A2780 cells at three of the time points, and maximal activity persisted through late log phase growth (day 6) before declining In addition, both GSH-Px and GSH-R activities peaked much earlier in the cisplatin-resistant line, by day 2, before leveling off or declining This pattern is similar to that of GSH in these cells Increased activities of these two enzymes in the resistant compared to the sensitive cells were only clearly evident on day 2, in early log phase growth, and may represent an enhanced response to the manipulations of passage If these manipulations result in some oxidative stress, it is possible that GSH-Px activity might be enhanced as a protective effect Given the larger GSH pool in these cells, increased GSH-Px activity could result in greater GSSG concentration, which would stimulate GSH-R activity

It is apparent that, to accurately compare the activity of these enzymes between different cells, the time from last passage must be controlled Erroneous conclusions could be made if this were not done For example, comparison of GSH-Px activity in 2780^{CP} on day 6 to that in A2780 cells on day 4 (60%), both in logarithmic growth, results in the exact opposite conclusion from the comparison of this enzyme in 2780^{CP} on day 4 to A2780 cells on day 6 (169%) Moreover, neither of these comparisons is consistent with comparing cells plated on the same day

It is evident that significant fluctuations in GSH and GSH-dependent enzymes during cell growth *in vitro* make meaningful comparison between different cell lines very difficult Besides differences in levels at any given time point, there may be differences in the pattern of fluctuations In these cells we have seen the greatest difference in pattern at an early post-passage time, possibly reflecting differences in response to passage manipulations Beyond this time point the differences appear smaller It is suggested that the optimal time point for comparison, if differences are sought among various cell lines, is in mid log phase growth Such fluctuations should also be kept in mind when comparisons are made of the relative cytotoxicity among different cell lines of an agent whose actions may be modulated by levels of cellular GSH or GSH-dependent enzymes

In summary, a number of recent studies have examined the level of glutathione and the activities of GSH-related enzymes in drug-sensitive and -resistant cell lines to determine their role in resistance to anticancer therapy We measured these variables serially over time since cell passage in a human ovarian cancer cell line derived from an untreated patient, and also in a subline selected for resistance to cisplatin *in vitro* We found significant fluctuations in the GSH level as well as in GSH-related enzyme activity over time The patterns of the fluctuations differed significantly between cell lines for some of these measurements Without adequate care to control for these fluctuations, comparison between cell lines could result in incorrect interpretation of the data

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Association and partitioning of propranolol in model and biological membranes

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Propranolol is a beta-adrenergic receptor blocking agent of considerable importance However, in addition to this specific therapeutic action, it also exerts a nonspecific action on membranes which is a membrane-stabilizing effect similar to that of other anesthetic molecules Membrane stabilization as a result of drug-membrane interaction is thought to be responsible for several of the pharmacological activities of propranolol [1] Previous attempts have been made to understand the mechanism of the propranolol–membrane interaction at the molecular level through *in vitro* experiments with model and biological membranes Thus, Godin *et al* [1] investigated the effects of propranolol on erythrocytes exposed to trinitrobenzene sulfonic acid and enzymes and concluded that reactivities were affected by perturbations in membrane protein as well as the phospholipid components Similar conclusions were drawn when propranolol was reacted with sarcoplasmic reticulum vesicles [2]. On studies with liposome systems, several investigators have reported a lowering of the phase transition temperature of the phospholipid by propranolol addition [3, 4] Also, propranolol has been found to displace Ca^{2+} from binding sites on phospholipid monolayers [5] The apparent partition coefficient of propranolol in negatively-charged liposomes has been found to be several times greater than in neutral liposomes [6, 7] Propranolol has been found to destabilize anionic liposomes at concentrations which provide an antihemolytic action on erythrocytes [8]

The relative importance of the drug–protein and the drug–phospholipid interactions is still not clearly understood for propranolol in its role as a membrane stabilizer Hence, studies of the uptake and partitioning of propranolol in erythrocytes, erythrocyte ghosts, liposomes, and the *n*-octanol–water system were aimed at obtaining additional evidence of the nature of these interactions

Materials and methods

DL-Propranolol hydrochloride was supplied by Ayerst Laboratories, Canada L-α-Dimyristoylphosphatidylcholine, 98% (DMPC) was obtained from the Sigma Chemical Co , St Louis, MO All other chemicals and solvents were reagent grade, and glass-distilled water was used

Studies with erythrocytes Fresh human blood was obtained from the University of Alberta Hospital for each series of experiments Ethylene-diamine-tetraacetic acid (EDTA) (0 12%) had been added as an anticoagulant Before each experiment, the blood was washed three times in isotonic Tris buffer, (15 mM hydroxymethylaminomethane, 50 mM NaCl, 5 mM glucose), pH 7 4, and then diluted to yield a test sample containing 5–7% hematocrit The test sample (2 ml) was diluted with a solution of the drug in Tris buffer (1 ml), divided into two equal portions and transferred to centrifuge tubes (Eppendorf), vortex-mixed, incubated at 37° for 15 min, and then centrifuged at 1500 rpm for 1 min The supernatant fraction was carefully removed, and its absorbance was measured at 288 nm (Pye Unicam SP6-550 spectrophotometer) against buffer as reference Absorbance values were corrected for the absorbance of a blank treated similarly Experiments with several blanks gave the same absorbance value verifying that the amount of absorbing material retained in the supernatant fraction was reasonably constant on all occasions Concentrations of propranolol were determined from a calibration curve and the association or uptake computed accordingly Concentration-dependent studies covered the range 0.1 to 2 3 mM propranolol Results are the averages of quadruplicate experiments

Studies with erythrocyte ghosts Erythrocyte ghosts were prepared from fresh human blood in the following manner Whole blood was centrifuged at 2500 rpm for 5 min and washed three times with normal saline solution The pooled, packed cells were diluted fourteen times with ice-cold isotonic Tris/EDTA solution (1 mM EDTA), mixed for 10 min, and then centrifuged (36,000 *g*, 37° 30 min) (Beckman L8-55 centrifuge) Subsequently, the supernatant fraction was removed, and the pellet was resuspended in Tris/EDTA and centrifuged again (36 000 *g* 37°, 15 min) Following this treatment the pellet was then washed twice in 10 mM Tris buffer and centrifuged as before Finally, the ghost cells were diluted back to the original volume, and this stock suspension was used for the uptake studies Normally, the test sample (4 ml of stock suspension) was diluted with isotonic, buffered drug solution (2 ml), vortex-mixed, incubated for 30 min, and then centrifuged (56,000 *g*, 37° 10 min) The uptake of propranolol by the ghost cells was determined from the residual concentrations of the supernatant fraction Triplicate experiments were run and the results averaged

Studies with liposomes Liposomes were prepared by the method of Bangham *et al* [9] A thin film of DMPC (50 mg) was formed on the wall of a round-bottom flask then dispersed in 5 ml of an isotonic phosphate buffer solution of propranolol at approximately 40°, and vortex-mixed for 10 min, then 3 5 ml was transferred to a centrifuge tube The liposomes were equilibrated at the desired temperature for at least 24 hr and then centrifuged (143,000 *g* 30 min), the concentration of drug in the supernatant fraction was determined spectrophotometrically Under these conditions, the residual phospholipid in the supernatant frac-