Heterogeneity of Glutathione Content in Human Ovarian Cancer


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ABSTRACT

Intracellular glutathione (GSH) has been shown to be one of the major factors modulating tumor response to a variety of commonly used antineoplastic agents. In this study the GSH contents of human ovarian tumors from primary biopsies, nude mouse xenografts, and in vitro cell cultures were compared. Pronounced intratumor cell-to-cell heterogeneity in GSH content was observed in primary patient biopsies when assessed using flow cytometry. For example, in an ascites biopsy from a newly diagnosed patient, a 5.6-fold difference in GSH concentration existed between the cell subpopulations with the 5% highest and 5% lowest GSH contents. Similar intratumor heterogeneity in GSH content was also evident in nude mouse xenografts. In addition, for a particular tumor line, the intertumor variations of GSH content among individual whole tumors were much less than the intratumor variation among slices from an individual tumor. Nude mouse xenografts of human ovarian cancer had GSH contents that were on average only slightly lower (30%) than those found in primary biopsies. In contrast, tumor cells grown as in vitro cultures, particularly those in exponential growth phase, had GSH contents considerably greater (1.3- to 3.5-fold) than those found in situ. Plateau phase cultures, however, had lower GSH contents and were more comparable to those observed in tumors in vivo. Overall, it may be concluded that in situations where GSH plays an important part in determining tumor response to a particular treatment, nude mouse xenografts may represent the most appropriate experimental model system.

INTRODUCTION

The effective treatment of epithelial ovarian cancer, a major cause of cancer fatalities among women in the United States, is still essentially out of reach. The current standard therapy of the disease emphasizes the combined modalities approach, in particular surgery and combination chemotherapy (1). However, relapse from treatment remains the rule rather than the exception. The causes of these failures have frequently been attributed to the development of drug resistance (2).

A number of diverse factors may account for the observed acquired refractoriness to chemotherapeutics. Prominent among these are: unfavorable changes in drug transport (3–5), increased DNA repair capacities (6–8), and increased protection by cellular detoxification systems, in particular the GSH system (7, 9–15). While it is now clear that P-glycoprotein-mediated reduction of intracellular drug concentration is an important mechanism of multidrug resistance in some tumor cell lines, numerous lines of evidence implicate mechanisms other than P-glycoprotein alone (for a recent review, see Ref. 16). For ovarian cancer, a great deal of research has focused on GSH itself. Using a series of human ovarian cell lines with acquired resistance to melphalan, cisplatin, and adriamycin, Ozols and co-workers (9, 14, 17) showed that both the primary resistance to one agent and the cross-resistance to other unrelated agents could be reversed by depleting GSH levels in tumor cells with BSO. It was further shown that reversal of resistance to melphalan by GSH depletion could be achieved in nude mice bearing human ovarian tumor xenografts (14). Additional support for GSH as a determining factor in drug resistance comes from our observation that the degree of reversal of drug resistance achieved by BSO is directly related to the extent of GSH depletion at the time of the chemotherapeutic agent exposure (10). Because of these promising developments, a Phase I clinical trial of BSO given in conjunction with an alkylating agent will soon begin at the National Cancer Institute, for the treatment of refractory ovarian cancer. Since the effectiveness of BSO depends directly on its ability to deplete GSH, it is of the utmost importance that any clinical protocol which includes this agent should also monitor the GSH content in tumors and critical normal tissues. This is of critical necessity because studies in mice have shown that tumors and normal tissues differ greatly in their GSH depletion and recovery following treatment with BSO (18, 19). However, the need for biopsies for GSH analyses is unlikely to be met in most tumor types, due to logistical constraints. Even in instances where biopsies can be obtained, the actual estimates of GSH content are likely to be confounded to variable degrees by contaminating normal cells (20). In this regard, ovarian cancer, because of the nature of its ascitic growth, offers an opportunity for obtaining biopsies for accurate measurements of GSH depletion in human tumors in situ. In this paper we present the first description of the GSH content of human ovarian cancer cells purified directly from primary patient ascites fluid by centrifugal elutriation. These data were then compared with those obtained for various laboratory human ovarian cancer model systems, viz. (a) tumor cell cultures at various phases of growth, (b) biopsies of in situ tumor xenografts grown in nude mice, and (c) tumor cells obtained from xenografts by centrifugal elutriation.

MATERIALS AND METHODS

Patients

Patients from whom specimens were obtained had, at the time of biopsy, Stage III or IV disease and diverse treatment history. Three patients had not received previous treatment. In some cases combination chemotherapy consisting of cyclophosphamide, Adriamycin, and cisplatin had been prescribed, while in others various second-line therapies had also been used.

Cell Lines

Seven tumor cell lines had been successfully established from biopsy specimens.

Tumor cells were purified from solid tumors, ascites, or cyst fluids by centrifugal elutriation. Cultures were initiated on bovine corneal matrix-coated dishes in α-minimum essential medium containing 10% fetal calf serum (JR Scientific, Woodland, CA) and 20% conditioned medium. A full description of the human ovarian adenocarcinoma cell lines used in this study, together with their response profiles to various chemotherapeutics (21) and radiation (22), have been reported.
OVARIAN CANCER GLUTATHIONE CONTENT

Cell Cultures

All cultures were maintained in α-minimum essential medium supplemented with 10% fetal calf serum, 5 mM glutamine, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Monolayer cultures in exponential growth were trypanized with 0.01% trypsin (Worthington)/0.02% EDTA. Single cells (5 × 10⁶) were placed in 100-mm Petri dishes. Cultures were transferred every 3–4 days.

Nude Mice and Xenografts

Intraperitoneal and s.c. xenografts were initiated by inoculation of 5 × 10⁶ cells from tissue culture or from elutriated primary specimens. Animals receiving i.p. implants developed ascites and multiple solid tumor masses on the ovaries, mesentary, and diaphragm. Subcutaneous xenografts grew as discrete solid masses and were used when they were at the size range of 200–800 mg. The histology of these xenografts was consistent with that of ovarian adenocarcinomas.

Centrifugal Elutriation

Ovarian solid and ascites tumors contain immune and connective tissue cells as well as tumor cells. Preparations of each cell type were isolated using a Beckman JE-6 elutriator rotor driven by a Beckman J21-C centrifuge. The elutriator and medium (α-minimum essential medium plus 10% normal bovine serum) were kept at 4°C at all times. After 10⁶ cells were loaded, subfractions were collected at decreasing rotor speeds. For each cell fraction, (a) the number of cells and the extent of tumor cell aggregation were determined using a hemocytometer, (b) the types of cells present were determined from Papanicolaou-stained cytocentrifuge preparations (23), and (c) cell volume was determined with a Coulter Channelizer, model ZBI, C1000. Since each patient used in this study had aneuploid tumor cells, flow cytometry was used as a second measure of tumor cell purity. Cells were fixed with 70% methanol and stained with acridine orange or propidium iodide, and the fluorescence intensity was quantitated on a Coulter EPICS V flow cytometer. The patient’s own lymphocytes served as the diploid control.

Glutathione Analysis

HPLC. The HPLC equipment consisted of Waters (Milford, MA) model 6000A chromatography pumps, model 710B automated sample processors (WISP), data module, model 720 system controller, Z-module, and model 420 fluorescence detector.

Glutathione was determined by an isocratic reversed phase paired-ion HPLC method, as described previously for tumor cells (10) and for solid tumor biopsies (19). Briefly, tumor cells (2–5 × 10⁶) were washed with phosphate-buffered saline and centrifuged at 400 × g for 10 min at 4°C and the cell pellet was stored at −70°C before analysis. Thawed cell pellets were homogenized with 200 μL, and frozen solid tumors with 20 volumes (w/v), of a 20 mM 5-sulfosalicylate solution and centrifuged in an Eppendorf microcentrifuge for 40 s. The supernatant was derivatized using the fluorescent reagent monobromobimane (Thiolyte; Calbiochem). The fluorescent GSH conjugate was eluted in a Waters Radial-PAC reversed phase bonded octadecylsane (C18) cartridge column (8-mm i.d.). Isocratic conditions at 3 ml/min, with a mobile phase consisting of 23% acetonitrile in 40 mM ammonium phosphate buffer, pH 7.2, containing 5 mM tetrabutylammonium hydroxide, were used. Fluorescence was detected at >410 nm with excitation at 340 nm. GSH concentrations were determined from peak heights with reference to a linear calibration curve that was constructed using synthetic GSH standards. Performance characteristics of this assay are typically: lower limit of detection, 8.0 pmol on column; coefficient of variation, 4–10%; accuracy, 91–111%.

Flow Cytometry. Single tumor cells from either in vitro cultures or mouse ascites fluid, at a concentration of 10⁶ cells/ml phosphate-buffered saline, were incubated with 20 μM monochlorobimane for 10 min at 25°C. Washed cells were then analyzed for fluorescence intensity with an EPICS V flow cytometer equipped with a krypton ion laser operated at 50–100 mW. Excitation wavelength was 350 nm and emission was detected at λ > 418 nm. Fluorescence intensity was found to increase with GSH content, as confirmed by HPLC.

Protein Assay

Soluble protein was estimated by the Bio-Rad assay (Richmond, CA) using bovine serum albumin, fraction V, as the standard.

RESULTS

Patient Primary Biopsies. Centrifugal elutriation separates cells, according to size and density, into 13 fractions. The nature of the cells in each fraction was assessed by RNA and DNA content analyses using flow cytometry (Fig. 1), as well as by Papanicolaou staining technique (23). The early fractions (fractions 1–4) usually contained purified immune cells. Fig. 1, A, C, and E, show the DNA and RNA histograms of fraction 4 from the ascites of a patient (GRA) with ovarian cancer, which contained 99% normal cells, over 80% of which were mesothelial cells. Fractions 5 to 8 contained mixtures of normal and tumor cells. Fractions 10 to 13 usually contained purified tumor cells. Cells were fixed with 70% methanol and stained with acridine orange or propidium iodide, and the fluorescence intensity was quantitated on a Coulter EPICS V flow cytometer. The patient’s own lymphocytes served as the diploid control.

Fig. 1. DNA and RNA histograms of elutriation-purified normal and tumor cells from the ascites fluid of a patient with ovarian cancer. Cells were stained with acridine orange. Red and green fluorescence are measures of tumor cell purity. Since considerable heterogeneity has been observed among different cell types in terms of cell volume and protein content, GSH level was expressed in three different units for the purpose of comparison between cell types. Also listed in Table 1 was the range of GSH content of normal immune cells purified in the early elutriated fractions. When normalized with respect to...
volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, macrophages, and mesothelial cells were found to be similar. In contrast, the tumor GSH values, listed as the mean values for volume and protein, the GSH contents of lymphocytes, mac...
which were tumor cells of similar sizes, a 5.6-fold difference in GSH existed between the cell subpopulations with the 5% highest and 5% lowest GSH contents. This degree of difference in GSH is sufficiently large to affect the sensitivity of some tumor cells to chemotherapy (13).

Nude Mice Xenografts. In our hands, about 50–60% of ovarian tumor cell lines established directly from patients formed xenografts in nude mice, either as asciates or solid s.c. tumors. Table 2 lists the GSH content of cells isolated by centrifugal elutriation from five different ascites tumors. Also shown are values obtained from solid s.c. OW-1 tumors following enzyme dissociation and centrifugal elutriation. The average GSH values for this series of xenografts were slightly less (70–76%) than those obtained for primary patient biopsies (Table 1).

GSH contents were also measured in solid tumor samples (Table 3). Four ovarian tumor lines were studied either as multiple (5–7) tumors of the same type, a 5.6-fold difference in GSH removal was observed between in vitro cell cultures and tumors grown in vivo. On average, on per cell, per volume, or per unit protein bases, cells in exponential growth in vitro had grown in different conditions (Table 1-4). Particularly striking disparities were observed between in vitro cell cultures and tumors grown in vivo. On average, on per cell, per volume, or per unit protein bases, cells in exponential growth in vitro had GSH contents 2.5-, 1.3-, and 3.5-fold higher, respectively, than cells obtained directly from patients. However, when in vitro cultures in stationary phase were used for comparison, these differences were greatly reduced, to 1.2-, 0.9-, and 2.2-fold, respectively.

These studies imply that in vitro sensitivity testing using exponential phase cultures may not accurately reflect the in vivo activities of antineoplastic agents such as cisplatin, cyclophosphamide, and melphalan, for which GSH plays a major detoxicative role. In addition, at least for ovarian cancer, human tumors in situ may be better represented by cultured cells in stationary phase of growth.

The cause for the decrease in cellular GSH content with decreasing proliferative state is not known, although the phenomenon itself is well established (20, 26–28). A deficiency in precursor supply appeared not to be a major factor, since daily growth medium replenishment could not halt the decline of cellular GSH. Nonproliferative state and reduction in GSH content can be induced by vastly different means including: cell-cell contact in the case of fed-plateau cultures, deficiencies of...
nutrients and growth factors in unfed-plateau culture (20), serum-deprived cultures (29), and lack of oxygen in hypoxic cells (27). It may be that decreased GSH contents in nonproliferating cells are more related to the physiological state of nonproliferation than to the growth conditions per se.

When ovarian tumor xenografts and primary patient tumor specimens were compared, the GSH concentrations of cells from these two sources were found to be very similar (Tables 1 and 2), the former having only slightly less GSH than the latter. The small differences between the two groups could simply be the consequence of the fact that they were originated from different patients. Compared to in vitro cell lines, xenografts resemble more closely human tumors in situ, with respect to such characteristics as growth conditions, nutrient supply, oxygen tension, intratumor heterogeneity, and contribution of drug metabolism to tumor response. In addition, the present results demonstrate that GSH determinations in nude mouse xenografts also resemble more closely measurements taken from patient ovarian cancers. Because of the importance of GSH in protecting cells against a variety of damaging agents including ionizing radiation and the majority of antineoplastic drugs, the desirability of this feature of the nude mouse xenografts in the testing of anticancer treatment may be significant. In light of the current observations, it is interesting to note that a recent report suggested that, provided stringent criteria for in vivo activity are followed, the use of xenografts in preclinical screening may be effective in identifying drugs with clinical activity for ovarian carcinoma (30).

Finally, there is the problem of obtaining patient tumor biopsies for GSH monitoring. In fact it appears that the measurement of GSH from just one tumor specimen will unlikely be truly representative of the tumor as a whole. By extension, an accurate assessment of the effects of an agent that changes GSH content, such as BSO, is unlikely to be achieved by the analysis of one biopsy specimen before and after treatment. This difficulty, caused by intratumor heterogeneity, will be alleviated to some extent if multiple biopsy specimens can be taken. Such a solution is, however, impractical for most tumor types. With BSO now heading for Phase I clinical trials, a precise method of monitoring its effects on GSH levels is clearly needed.

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REFERENCES

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