Radiation Survival Parameters of Antineoplastic Drug-sensitive and -resistant Human Ovarian Cancer Cell Lines and Their Modification by Buthionine Sulfoximine

Karen G. Louie, Brent C. Behrens, Timothy J. Kinsella, Thomas C. Hamilton, Karen R. Grotzinger, Wilma M. McKoy, Margaret A. Winker, and Robert F. Ozols

ABSTRACT

The optimum integration of chemotherapy and irradiation is of potential clinical significance in the treatment of ovarian cancer. A series of human ovarian cancer cell lines have been developed in which dose-response relationships to standard anticancer drugs have been determined, and the patterns of cross-resistance between these drugs and irradiation have been established. By stepwise incubation with drugs, sublines of A2780, a drug-sensitive cell line, have been made 100-fold, 10-fold, and 10-fold more resistant to Adriamycin (2780AD), melphalan (2780ME), and cisplatin (2780CP). Two additional cell lines, NIH:OVCAR-3AD(A9+) and NIH:OVCAR-4AD(A9+), were established from drug-refractory patients. 2780ME, 2780CP, OVCAR-3AD(A9+), and OVCAR-4AD(A9+) are all cross-resistant to irradiation, with D0s of 146, 187, 143, and 203, respectively. However, 2780AD remains sensitive to radiation, with a D0 of 111, which is similar to that of A2780 (101). Glutathione (GSH) levels are elevated in 2780ME, 2780CP, OVCAR-3AD(A9+), and OVCAR-4AD(A9+) to 4.58, 6.13, 12.10, and 15.14 nmol/10⁶ cells as compared to A2780, with 1.89 nmol/10⁶ cells. However, the GSH level in 2780AD is only minimally higher than that in A2780 (2.94 nmol/10⁶ cells). Buthionine sulfoximine, a specific inhibitor of GSH synthesis, significantly increases the radiation sensitivity of 2780ME (changing the D0 from 143 to 95) and 2780CP to a lesser extent, suggesting that intracellular GSH levels may play an important role in the radiation response of certain neoplastic cells. These results suggest that the sequential use of irradiation following chemotherapy with melphalan and cisplatin may be less effective than a combined modality approach, which integrates radiation and chemotherapy prior to the development of drug resistance and cross-resistance to irradiation.

INTRODUCTION

Most patients with ovarian cancer have advanced disease (FIGO Stage III to IV) at the time of diagnosis (10). Combination chemotherapy regimens have produced clinical complete responses in 40 to 50% patients with advanced disease, and second-look laparotomies have demonstrated that the pathological complete response rate is 20 to 30% (5, 14, 44, 46, 47). Whole abdominal irradiation has also been demonstrated to be effective therapy in patients with small volume residual disease (11, 12). In an effort to increase the complete response rate, combined modality approaches with radiation and chemotherapy are currently under investigation at the National Cancer Institute and elsewhere (18, 24, 28). The designs of these clinical trials integrating irradiation and chemotherapy have been empirically derived, and the preliminary results of most studies have failed to demonstrate a marked advantage over the use of combination chemotherapy alone (24, 28). Experimental studies on patterns of radiation and chemotherapeutic sensitivity in relevant model systems of ovarian cancer may be useful in optimizing treatment schedules.

Recently, several ovarian cancer cell lines have been established, both from previously untreated patients and from patients clinically resistant to chemotherapy (2, 22). NIH:OVCAR-4AD(A9+) and NIH:OVCAR-3AD(A9+) were derived from patients who had become refractory to cyclophosphamide, cisplatin, and Adriamycin. In addition, by stepwise incubation of A2780 (a sensitive cell line cloned from an untreated patient) with drugs, variant cell lines with resistance to Adriamycin (2780AD), melphalan (2780ME), or cisplatin (2780CP) were established (16, 22). The sublines are approximately 100-fold, 10-fold, and 10-fold more resistant to the respective drugs than is the parent line (A2780). These cell lines have been used to determine dose-response relationships to chemotherapy, to study the radiosensitivity of human ovarian cancer cells, and to establish the pattern of cross-resistance to irradiation in cell lines with primary resistance induced against antineoplastic drugs. 2780ME is 3-fold more resistant to Adriamycin than is A2780, and 2780AD is 2.5-fold more resistant to melphalan than is the parent line (2). In addition, these cell lines may be useful for evaluating the role of radiation and chemosensitizing agents such as BSO. This drug, a specific inhibitor of glutathione synthesis, has been shown to markedly depress the intracellular GSH level and enhance both radiation and chemosensitivity of many cell types (7, 13, 19–21, 29, 39). The results of these studies may have direct relevance to the design of clinical trials aimed at evaluating combined modality therapy in ovarian cancer.

MATERIALS AND METHODS

Chemicals and Reagents. RPMI 1640, fetal bovine serum, penicillin/streptomycin, and glutamine were obtained from Grand Island Biological Company, Glen Burnie, Maryland. Buthionine sulfoximine; GSH, glutathione; IC50, drug concentration which inhibits 50% colony growth; Do, slope of the exponential portion of the curve.
Company, Chagrin Falls, OH. Insulin (Iletin U-100) was from Eli Lilly & Company, Indianapolis, IN. d-L-Buthionine-S-R-sulfoximine was from Chemalog (Chemical Dynamics Corporation), South Plainfield, NJ. Type VII agarose was obtained from Sigma Chemical Company, St. Louis, MO.

Tissue Culture Medium. Cells were passaged and maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, insulin (0.25 U/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (0.3 mg/ml) as described previously (32). Cells were incubated at 37°C in a humidified atmosphere of 5% (v/v) CO₂ and medium was changed every 3 to 4 days.

Clonogenic Assay. Drug sensitivity and radiation survival curves were determined using clonogenicity in soft agarose as described previously (32). In brief, cells were harvested with a trypsin (0.05%, w/v)/EDTA (0.02%, w/v) solution and counted with a Coulter counter (Coulter Electronics model ZBII). Cells in single cell suspension were plated in a mixture of 0.3% (w/v) agarose and RPMI 1640 (including the ingredients listed above) over a layer of solidified 0.6% (w/v) agarose in 10-sq cm dishes. A2780 and its variants were plated at a concentration of 10,000 to 15,000 cells/dish, yielding cloning efficiencies of 20 to 45%. OVCAR-4(A8+) had a cloning efficiency of 4%, with 20,000 cells/dish, and OVCAR-S7 had a cloning efficiency of 1%, with 75,000 cells/dish.

Radiation. Cells growing exponentially were plated in 10-sq cm dishes as described above. At 4 to 6 h after trypsinization, cells were irradiated at room temperature using a 15-MeV photon beam from a Varian 20 linear accelerator. Dishes received graded doses of radiation from 50 to 600 rads at a dose rate of 500 rads/min. Dosimetry was carried out using a Baldwin-Farmer ionization chamber connected to a Keithly electrometer system having direct National Bureau of Standards calibration. Full electron equilibrium was insured for all radiation. Dishes were incubated for 1 week at 37°C in a humidified 5% (v/v) CO₂ atmosphere. Colonies measuring greater than 60 μm and containing greater than 50 cells were counted on a Bausch and Lomb Omnicon FAS II system. Using the linear portion of the curves, a least squares regression analysis of the individual dose data points for each cell line was determined to generate radiation survival curves. At least 2 experiments were conducted on each cell line. The radiation curves are characterized by a shoulder at low doses, merging into an exponential line at higher doses on a semilogarithmic plot. Conventionally, two parameters have been used to describe the radiation survival curve: D₀, the slope of the exponential portion of the curve (the dose required to reduce survival to 0.37 on any survival level of the exponential portion of the curve) and Dₙ or n (extrapolation number), which is a measure of the width of the shoulder of the curve and which is calculated by extrapolating the straight portion of the curve back to the surviving fraction axis. Multiplicity at the time of irradiation was assumed to be unity.

BSO Treatment. A2780 and its variants growing in log phase were pretreated in monolayer culture with 25 μM BSO for 48 h prior to cloning. BSO 25 μM was also added to the upper agarose-cell suspension before plating. Both control and BSO treated plates were fed with growth medium, without BSO, on Day 4 and scored for colony growth on Day 10. NIH:OVCAR-3(A8+) and NIH:OVCAR-4(A8+) were treated similarly with 15 μM BSO. Doses of BSO which were minimally cytotoxic were selected for the individual cell lines. For 2780ME and 2780CP, treatment with 25 μM BSO resulted in 70 to 95% survival compared to untreated controls. In OVCAR-3(A8+) and OVCAR-4(A8+), incubation with 15 μM BSO produced survival of 45% compared to untreated controls.

Measurement of GSH. Five × 10⁶ cells from each line both with and without prior BSO treatment in monolayer were centrifuged at 10°C, 300 × g, for 10 min. The pellet was resuspended in 5 ml saline and centrifuged again as above. Total GSH levels were measured a minimum of 3 times by the method of Tietze after preparation of a protein-free filtrate (41). GSH levels of all other cell lines were compared to A2780 by a 2-tailed t-test.

RESULTS

Characteristics of Ovarian Cancer Cell Lines. By stepwise incubation with individual drugs, variants of a cell line derived from an untreated ovarian cancer patient (A2780) have been developed with resistance to melphalan, Adriamycin, or cisplatin (22). 2780AD was exposed to gradually increasing doses of Adriamycin over a 6-month period up to a maximum dose of 2 μM and then grown in the absence of drug for more than 6 months without loss of resistance to the induction drug. 2780ME and 2780CP were treated similarly with maximum drug concentrations of 10 and 8 μM, respectively. The doubling times for the parent line, as well as the variants, are approximately 14 h. Cross-resistance patterns to chemotherapeutic agents have been described previously (2). 2780ME is partially cross-resistant to Adriamycin, and 2780AD is partially cross-resistant to melphalan (2).

NIH:OVCAR-4(A8+) and NIH:OVCAR-3(A8+) were obtained from patients who were clinically refractory to cyclophosphamide, Adriamycin, and cisplatin at the time the lines were established (23). The doubling time of OVCAR-4(A8+) is 25 h, and that of OVCAR-3(A8+) is 63 h. The inherent resistance of OVCAR-3(A8+) and OVCAR-4(A8+) to melphalan is the same order of magnitude as 2780ME; however, 2780AD is much more resistant to Adriamycin than either OVCAR-4(A8+) or OVCAR-3(A8+) (Table 1). The IC₅₀ (drug concentration which inhibits 50% colony growth) for melphalan is 5.5 μM in 2780ME as compared to 1.5 μM in OVCAR-3(A8+) and 1.4 μM in OVCAR-4(A8+). The results for Adriamycin show an IC₅₀ of 1.6 μM in 2780AD versus 0.02 μM and 0.06 μM for OVCAR-3(A8+) and OVCAR-4(A8+), respectively.

Radiation Studies. Radiation survival curves for A2780 and the variant drug resistant cell lines are shown in Chart 1. The D₀ for A2780 is 101, while the n is 1.40. These results are similar to those of other tumor cell lines, both nonhuman and human, reported in the literature (17, 45). 2780ME has a D₀ of 146 and an n of 2.12. 2780CP has a D₀ of 187 and an n of 1.82. These two lines demonstrate a statistically significant increase in resistance to irradiation. In contrast, 2780AD has a D₀ of 111 with an n of 1.48, values which are not statistically different from those of the parent line (A2780).

The radiation survival curves for OVCAR-4(A8+) and OVCAR-3(A8+) are shown in Chart 2. The D₀s of 203 and 143 and n's of 1.08 and 1.53 demonstrate radiation responses similar to 2780ME and 2780CP.

GSH Levels and the Effect of Buthionine Sulfoximine. Total intracellular GSH levels in A2780 and its variants, as well as in OVCAR-3(A8+) and OVCAR-4(A8+), were determined at the time of irradiation. The results are shown in Table 2. Of A2780 and

Table 1

<table>
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<th>Cell line</th>
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<th>Adriamycin</th>
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</tr>
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RADIATION DOSE (rads)

Chart 1. Radiation survival curves for A2780 and its resistant variants: (C) A2780; (2) 2780<sup>AD</sup>; (4) 2780<sup>ME</sup>; and (•) 2780<sup>CP</sup>. Cells were plated in 0.3% agarose and radiated at doses of 50 to 600 rads. Colonies larger than 60 μm in size were scored on Day 7. Each point represents the mean of at least 4 experiments. Curves were generated using linear regression by least squares analysis. By Student's t test, 2780<sup>AD</sup> and 2780<sup>CP</sup> are statistically different from A2780 (P < 0.01). 2780<sup>ME</sup> does not differ significantly from A2780. Bars, SE.

its variants, 2780<sup>ME</sup> and 2780<sup>CP</sup> have the highest levels of GSH, with 4.58 and 6.13 nmol/10<sup>6</sup> cells, respectively. In contrast, 2780<sup>AD</sup> has a level of 2.94 nmol GSH/10<sup>6</sup> cells, which is only 50% higher than that of the parent line (1.89 nmol/10<sup>6</sup> cells). OVCAR-3<sup>4(Ag+)</sup> and OVCAR-4<sup>4(Ag+)</sup> have the highest intracellular GSH levels, with 12.10 and 15.14 nmol/10<sup>6</sup> cells, respectively.

Preincubation (48 h) with 25 μM BSO in A2780 and its variants (2780<sup>CP</sup>, 2780<sup>AD</sup>, and 2780<sup>ME</sup>) resulted in a decrease of intracellular GSH to less than 0.75 nmol/10<sup>6</sup> cells (Table 2). The 48-h preincubation with 15 μM BSO in OVCAR-3<sup>4(Ag+)</sup> and OVCAR-4<sup>4(Ag+)</sup> resulted in intracellular GSH levels which were greater than 2.0 nmol/10<sup>6</sup> cells (Table 2).

Radiation Sensitization by BSO. Depletion of intracellular GSH by 48-h monolayer exposure to BSO followed by maintenance of the GSH-depleted state by the presence of BSO in the cloning medium, i.e., during irradiation and for 4 days thereafter, caused a marked sensitization in 2780<sup>ME</sup>, with the D<sub>0</sub> decreasing from 143 to 95 (Chart 3). Similar treatment of 2780<sup>CP</sup> resulted in less sensitization to radiation, with the D<sub>0</sub> changing from 183 to 134 (Table 3). However, no significant change in n values was seen for these two lines.

Incubation of OVCAR-3<sup>4(Ag+)</sup> with 15 μM BSO resulted in only a small change in D<sub>0</sub> but a significant change in n. In contrast, BSO treatment of OVCAR-4<sup>4(Ag+)</sup> did not result in radiosensitization (Table 3).

DISCUSSION

The development of resistance and cross-resistance to chemotherapy and irradiation is a major clinical problem in the treatment of ovarian cancer. Dose-response relationships and patterns of cross-resistance in established ovarian cancer cell lines...
have not been fully evaluated. However, possibilities include altered drug transport, changes in the binding or cross-linking to DNA, changes in the repair of DNA, and metabolism to less toxic products (9, 15, 33, 35, 37, 40, 50). While less is known regarding the action of cisplatin, it is thought to form interstrand cross-links with DNA in a manner similar to melphalan (48–50). Thus, melphalan and cisplatin have direct effects on cellular DNA, causing interstrand cross-links, and resistance to these drugs leads to cross-resistance with irradiation.

In contrast, the mechanism of action of Adriamycin is least understood. Adriamycin-resistant cells in some experimental systems have been found to accumulate less drug, which appears to be caused either by an increased rate of active drug efflux or by changes in the lipid structural order of the cell membrane (25, 26, 31, 34, 36, 38, 42, 43). It is felt that the cardiac toxicity of Adriamycin is mediated via free radical damage; however, it is not known whether tumor cell cytotoxicity is similarly mediated (1, 30). It is of considerable interest therefore that Adriamycin-resistant cells do not develop cross-resistance to irradiation.

Clinical trials combining combination chemotherapy and subsequent radiation are currently undergoing evaluation (24, 28). Preliminary results suggest that irradiation confers no additional benefit over combination chemotherapy alone. The results of our study demonstrate that cross-resistance to radiation develops in melphalan- and cisplatin-resistant variant cell lines as well as in lines established from clinically drug-refractory patients. These findings may provide an explanation for the failure of combined modality regimens that utilize radiation after combination chemotherapy. The optimum regimen might not be chemotherapy (with cisplatin and alkylating agents) followed by irradiation, but perhaps simultaneous radiation and chemotherapy.

Intracellular thiols, such as glutathione, are thought to be involved either in protection from radiation-induced damage or in the immediate reduction of free radicals formed by irradiation (4, 6, 7, 29). Agents which lower the level of intracellular GSH have also been shown to increase the radiation sensitivity of some cell lines but not others (3, 7, 13, 24). However, ataxia telangiectasia cell lines which have a markedly increased sensitivity to killing by X-rays have normal GSH levels (27). Elevated intracellular GSH levels have been found both in melphalan-resistant L1210 leukemia cells and in melphalan-resistant lines of human ovarian cancer (19, 39). 2780ME and 2780CP both have 2.5 to 3.5 times the level of intracellular GSH found in A2780.

2780CP, on the other hand, had a smaller GSH elevation (approximately 1.5-fold). As expected, the two sublines with higher GSH levels (2780ME + 2780CP) are more radiosensitive than those sublines with lower GSH (A2780 + 2780CP). Additionally, OVCAR-3ICo and OVCAR-4ICo, from patients refractory to cyclophosphamide, Adriamycin and cisplatin, also have very high GSH levels and are quite radioresistant. These findings are in agreement with the theory that intracellular thiols such as GSH are intimately involved in protection from radiation damage (7, 29).

BSO is a specific inhibitor of the enzyme γ-glutamyl cysteine synthetase, which is required for GSH synthesis (20, 21). Melphalan-resistant L1210 leukemia cells (which have elevated base line GSH levels), when treated with BSO, developed markedly decreased levels of GSH and increased sensitivity to melphalan (39). In human ovarian cancer cell lines, BSO has also been shown to completely reverse melphalan resistance (19). In this
study, we have now demonstrated that decreasing the level of intracellular GSH with BSO can completely restore radiation sensitivity to 2780CP. Despite GSH levels which were decreased to the same extent as in 2780CP (0.75 nmol/10^6 cells), BSO treatment in 2780CP caused less sensitization to irradiation. This suggests that the elevation of intracellular GSH may not be the only mechanism involved in the development of cross-resistance to irradiation in cells with primary resistance induced to cisplatin, in contrast to the apparent situation in which primary resistance is induced with melphalan.

Because of the increased cytotoxicity of BSO in the OVCAR cell lines, OVCAR-3^{44/9} and OVCAR-4^{44/9} were treated with 15 μM BSO. At this dose level, total GSH could not be depressed below 2.0 nmol/10^6 cells (a level higher than the inherent level of A2780). The observation that BSO treatment had no effect on the radiation survival parameters of OVCAR-3^{44/9} and OVCAR-4^{44/9} suggests that there may be a threshold below which GSH levels must be depressed in order to produce radiation sensitization.

While it is clear that cells with elevated intracellular GSH levels are more resistant to ionizing radiation and that reduction of GSH levels (via specific enzyme inhibition) can lead to reversal of that resistance, the molecular mechanisms involved have not been established. Possible explanations for GSH protection from radiation-induced damage include the immediate reduction of free radicals both on DNA itself and on other radiolytic products, as well as modifications of DNA repair enzymes or their environment to enhance activity. Studies to evaluate these possibilities as they relate to both radiation and chemotherapeutic drug resistance are in progress.

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