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Chemotherapy of Ovarian Cancer Directed by the Human Tumor Stem Cell Assay

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Summary. *The human tumor stem cell assay (HTSCA) has been used to study the in vitro sensitivity rates of anticancer drugs used in the treatment of 115 patients with previously untreated and relapsing ovarian cancer. The data from these studies have identified patterns of cross resistance and residual sensitivity between these agents, and have allowed the prospective selection of single agents possessing in vitro activity for the treatment of 32 patients with relapsing disease. cis-Platinum and vinblastine were the most active agents in vitro against ovarian TCFUs from both previously untreated and relapsing patients. Prior therapy with even one drug was associated with the acquisition of resistance to several classes of compounds (e.g., melphalan resistance was almost always associated with in vitro adriamycin resistance, $P < 0.001$). A clinical trial yielding similar data would have required nearly 450 evaluable ovarian cancer patients. In 11 of 32 patients in vitro testing predicted sensitivity to single agents: eight of these had partial remissions for a predictive accuracy of 73%. In 33 instances the HTSCA had 100% accuracy in predicting the lack of clinical response. Thus, the HTSCA for advanced ovarian cancer appears to have a similar predictive accuracy rate to the estrogen receptor assay for predicting the response to hormonal therapy for disseminated breast cancer.*

Introduction

There are at least a dozen anticancer drugs which are active in the treatment of patients with advanced ovarian cancer of epithelial type [6, 22, 26]. In the past chemotherapy has been selected on an empirical basis, with as many as three or more agents being combined for the treatment of newly diagnosed patients with stages III and IV disease [9, 25, 27]. When patients relapse following therapy with multiple-drug regimens the response rate to single agents is in the range of 0–25% [12, 21, 28]. Such low response rates may result from the apparent development of cross resistance between different classes of compounds [1] and the heterogeneity of responsiveness of tumors to different drugs.

Using the human tumor stem cell assay (HTSCA) [10, 11, 20], we have studied the in vitro sensitivity rates of anticancer drugs used in the treatment of patients with previously untreated and relapsing ovarian cancer. The data from these studies have identified patterns of cross sensitivity and resistance between these agents [1], and have allowed the prospective selection of active agents (i.e., sensitive in vitro) for the treatment of relapsing disease [2, 3].

Methods

Patients. In vitro drug sensitivity assays were performed on 115 women with surgically and histologically proven ovarian adenocarcinoma of epithelial type. Twenty-nine of these patients had had no prior chemotherapy, and 86 had received previous anticancer drug treatment. No patient had received chemotherapy within 3 weeks of drug assay. Of these 115 patients, 32 have since undergone 44 clinical trials with single agents that were tested in vitro. All 32 of these patients had clinically measurable disease, which had recurred following chemotherapy. All correlations between in vitro and in vivo drug sensitivity were made prospectively, whereas correlations of drug resistance were made both prospectively and retrospectively. The clinical data for 31 of these 32 patients have been published previously [3, 20]. Standard criteria were used to evaluate objective response. Partial remissions were defined as at least 50% reduction in the size of all measurable tumor masses for longer than 1 month.

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In vitro Clonogenic Assay. Tumor samples for culture were obtained from both solid tissues and malignant pleural and peritoneal effusions. Techniques for preparing single-cell suspensions, for drug incubations, and for plating the cells in the 'tumor stem cell' agar cultures were as reported previously [10, 11, 20], except that conditioned medium was not used, since sufficient ovarian tumor colony growth (e.g., 30–200 colonies per 35-mm petri dish) was usually obtained within 7–10 days without conditioning. For the drug assay, cells were exposed to varying concentrations of drugs in tissue culture tubes for 1 h at 37° C before they were washed and plated [20]. Standard agents for in vitro drug testing included melphalan, adriamycin, *cis*-platinum, methotrexate, 5-fluorouracil, vinblastine, vincristine, bleomycin, and carmustine (BCNU), which were all tested at low doses up to an upper limit of pharmacologically achievable concentrations [4, 16]. Freshly plated cultures were examined by inverted light microscopy to ascertain that aggregates were not present. Plates were cultured under standard assay conditions. Clusters (15–30 cells) apparent within 3 days and colonies (30-cell aggregates) were usually present in sufficient numbers and size to be counted by inverted microscopy with the Bausch and Lomb FASII 7–10 days after plating.

Representative plates were prepared for morphological analysis with a dried slide technique that involved Papanicolaou staining [19].

Data Analysis. Data from all in vitro experiments were stored in a laboratory computer, which was used for data analysis and graphic output. The statistical comparison of populations was carried out by methods for the analysis of contigency tables [5].

Interpretation of in vitro sensitivity to standard drugs was based on the area under linear survival concentration curves (i.e., sensitivity index), obtained in a group of 96 patients who had been studied earlier [16]. Tumor stem cell assays and drug sensitivity measurements on biopsy samples from these patients showed that, overall, tumor colony forming units (TCFUs) from about 75% of ovarian tumor samples undergo in vitro clonogenic growth in the culture system and provide sufficient colony growth to permit assessment of drug-induced lethality [3, 11, 20]. Ovarian cancers were considered to be sensitive to a drug in vitro if the sensitivity index for specific anticancer drugs were less than the following relative area units: 5.3 for melphalan, 9.5 for adriamycin, 11.4 for *cis*-platinum, 6.5 for methotrexate, 7.5 for 5-fluorouracil, 3.8 for bleomycin, 6.4 for vincristine, and 10 for mAMSA, BCNU, vinblastine, and vindesine [16]. Tumor stem cells were considered sensitive to 13-*cis*-retinoic acid if reduced to less than 30% of control values at 10^{-9} *M* exposure in vitro by continuous contact. This plasma concentration is readily achievable with daily oral dosing of this agent.

Results

Patterns of Anticancer Drug Cross Sensitivity and Resistance

Shown in Table 1 are the frequencies of in vitro sensitivity to standard drugs of ovarian cancer stem cells from untreated versus previously treated patients. Note that *cis*-platinum had the highest sensitivity rate (i.e., 64%) of drugs tested against tumor stem cells from previously untreated patients. On the other hand, melphalan was quite ineffective, a finding which will be discussed later. The in vitro sensitivity rates for these drugs dropped significantly for *cis*-platinum ($P = 0.01$), adriamycin ($P = 0.05$), and bleomycin ($P = 0.10$) following in vivo exposure to any drug therapy, but vinblastine usually manifested activity despite prior therapy with other agents.

In the previously untreated patients there was evidence for in vitro cross resistance between melphalan and adriamycin and between melphalan and bleomycin. Of the 28 previously untreated patients who were tested in vitro for adriamycin sensitivity (Table 1), 20 were resistant and eight sensitive in vitro to melphalan. Of the 20 melphalan-resistant patients only three (15%) were sensitive to adriamycin, whereas all eight melphalan-sensitive patients were also sensitive to adriamycin (100%) ($P < 0.001$ for difference in adriamycin sensitivity rates). Similar data apply to bleomycin-melphalan in vitro cross resistance. Of 13 melphalan-resistant patients only two (15%) were sensitive to bleomycin, whereas four of five (80%) melphalan-sensitive patients were also sensitive to bleomycin ($P < 0.01$).

In relapsing ovarian cancer patients vinblastine was as active as *cis*-platinum in the inhibition of TCFUs ($P > 0.20$) (Tables 2 and 3). The sensitivity rates to vinblastine ranged between 19% and 36% (mean 26%) for these patients. Vinblastine and *cis*-platinum did not appear cross-resistant, 25% of the tumors being sensitive to the former agent in the setting of in vitro resistance to the latter. On the other hand, vinblastine did appear to be cross-resistant ($P = 0.44$) with the related vinca alkaloid vindesine. None of ten ovarian cancers resistant to vinblastine was sensitive in vitro to vindesine.

In vitro-in vivo Tumor Sensitivity Correlations

The relation between results of in vitro drug sensitivity assays and outcome of treatment by anticancer drugs in 32 patients is summarized in Table 4. Thirty-one of these 32 patients have been reported on previously [2, 3, 20], including patients 1–7 in Table 5. In 11 of the 32 patients, in vitro testing predicted sensitivity to single agents; eight of these had partial remissions lasting a median of 3.3 months (Table 5). Three patients whose tumors were sensitive to specific anticancer drugs in vitro failed to respond clinically to these same drugs (false-positive assays). Thus, the predictive accuracy of the in vitro assay for objective response was 73% ± 13.4% (i.e., 8 of 11). Responses were predicted for such occasionally used single agents as 13-*cis*-retinoic acid, vindesine, bleo-

Table 1. Frequency of in vitro drug sensitivity[a] of TCFUs from previously untreated versus relapsing ovarian cancer patients

Drug tested	Untreated patients			Relapsing patients		
	Sensitive	Total	%	Sensitive	Total	%
cis-Platinum	16	25	64	18	65	28
Vinblastine	7	17	41	23	66	35
Adriamycin	11	28	39	11	56	20
Bleomycin	6	18	33	9	57	16
Melphalan	3	23	13	8	50	16

[a] For definition see *Methods* and references [4, 16, 20]

Table 2. Frequency of sensitivity to *cis*-platinum by TCFUs 'resistant' to standard agents[a]

Resistant drug	Total number tested	Number sensitive to *cis*-platinum	Sensitivity % (SE)
Adriamycin	40	7	18 (6.1)
Bleomycin	38	11	29 (7.4)
mAMSA	11	3	27 (13.4)
Melphalan	37	6	16 (6.0)
Methotrexate	18	3	17 (8.9)
Vinblastine	34	7	21 (7.0)

[a] Data on TCFUs from 86 patients in relapse are included in this analysis

Table 3. Frequency of sensitivity of vinblastine by TCFUs 'resistant' to standard agents[a]

Resistant drug	Total number tested	Number sensitive to vinblastine	Sensitivity % (SE)
Adriamycin	36	10	28 (7.5)
Bleomycin	36	13	36 (8.0)
cis-Platinum	36	9	25 (7.2)
mAMSA	15	5	33 (12.1)
Melphalan	30	9	30 (8.4)
Methotrexate	16	3	19 (9.8)

[a] Data on TCFUs from 86 patients in relapse are included in this analysis

Table 4. Correlation of in vitro sensitivity and clinical response in 32 patients with ovarian cancer

Number of patients	Number of clinical trials	Sensitive in vitro	Sensitive in vitro	Resistant in vitro	Resistant in vitro
		Sensitive in vivo	Resistant in vivo	Sensitive in vivo	Resistant in vivo
32	44	8 (True-positives)	3 (False-positives)	0 (False-negatives)	33 (True-negatives)

Total no. of trials: 44

Predictive accuracy for sensitivity $= \frac{8}{11} \times 100 = 73\%$ $(P < 0.0001)$

Predictive accuracy for resistance $= \frac{33}{33} \times 100 = 100\%$ $(P < 0.0001)$

mycin, and vinblastine, as well as the more commonly used adriamycin, melphalan, and *cis*-platinum (Table 5). Bleomycin's activity against the TCFUs from patient 2 is shown in Fig. 1. Note that melphalan and adriamycin had little activity at low in vitro concentrations. This patient had complete disappearance of malignant ascites for a period of 4 months following IP administration of bleomycin at 60 U/m^2 for two doses. When patient 2's ascites recurred her TCFUs were again tested for bleomycin sensitivity. In Fig. 2

Table 5. Characteristics of patients with ovarian cancer whose tumor regressions were predicted by in vitro drug assay

Patient number	Previous therapy	In vitro drug resistance	In vitro drug sensitivity	Treatment	Response	Duration (months)	Response description
1	Adriamycin-cyclophosphamide	Melphalan Bleomycin	Vinblastine	Vinblastine, 7 mg/m^2 every week	PR	3	Reversal of bowel obstruction; 50% decrease in pelvic mass
2	Actinomycin D-5-fluorouracil-cyclophosphamide; Adriamycin-*cis*-platinum – hexamethylmelamine	Melphalan BCNU	Bleomycin	Bleomycin, 60 U/m^2 IP × 2	PR	4	Complete disappearance of malignant ascites
3	Cyclophosphamide-methotrexate-5-fluorouracil	Melphalan Bleomycin Adriamycin Methotrexate *cis*-Platinum	Vinblastine	Vinblastine, 7 mg/m^2 every week	PR	5	Complete disappearance of malignant pleural effusions
4	Adriamycin-cyclophosphamide-bleomycin	Adriamycin *cis*-Platinum Methotrexate	Vindesine	Vindesine, 3 mg/m^2 every week	PR	2	Complete disappearance of malignant ascites
5	Melphalan	Melphalan *cis*-Platinum Vinblastine	Adriamycin	Adriamycin, 60 mg/m^2 every 3 weeks	PR	2	Greater than 50% decrease in intra-abdominal tumor mass
6	Adriamycin-cyclophosphamide; Melphalan; Bleomycin; *cis*-Platinum	Melphalan Methotrexate mAMSA Pentamethyl-melamine	13-*cis*-Retionic acid	13-*cis*-Retinoic acid, 1 mg/kg PO daily	PR	2	Complete disappearance of malignant ascites and greater than 33% decrease in pelvic mass
7	Adriamycin-cyclophosphamide; *cis*-Platinum-5-fluorouracil-hexamethyl-melamine	Vinblastine Methotrexate Mitomycin C mAMSA	Melphalan	Melphalan, 1 mg/kg IP followed by 1 mg/kg PO every 4 weeks	PR	5	Complete disappearance of malignant ascites and greater than 90% decrease in pelvic mass
8	Melphalan	Adriamycin Melphalan Vinblastine MGBG	*cis*-Platinum	*cis*-Platinum, 50 mg/m^2 every 3 weeks	PR	3	Complete disappearance of malignant ascites

is shown the complete resistance to bleomycin of the patient's TCFUs, which correlated with lack of clinical response to further IP dosing.

Among the 32 patients there were 33 instances of lack of clinical response successfully predicted by in vitro drug resistance (true-negatives). Since there were no instances of in vitro resistance being associated with in vivo sensitivity to the same drug, the accuracy of the assay for predicting lack of clinical response was 100%. The statistical test of association between all the in vitro and in vivo study results was highly significant ($P < 0.0001$).

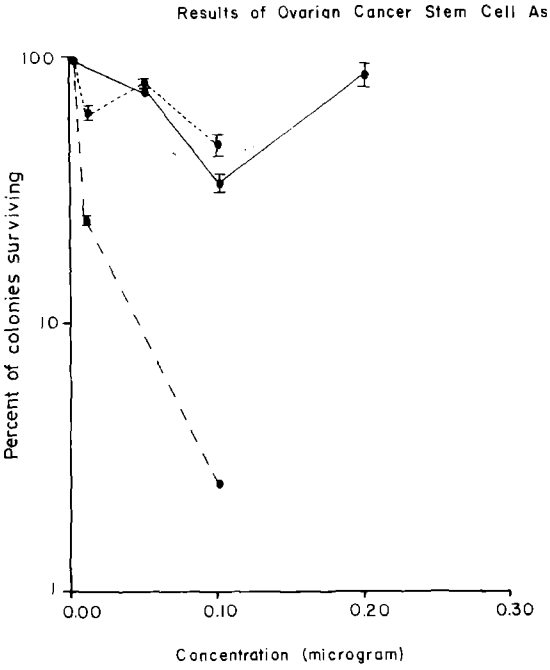

Fig. 1. The effect of melphalan, adriamycin, and bleomycin on the inhibition of ovarian TCFUs of patient 2 (Table 5) in February, 1977. Note the steep dose-survival curve resulting from bleomycin exposure (●– – –●). (———) Melphalan; (- - - -) Adriamycin; (– – –) Bleomycin

Discussion

This analysis of drug cross sensitivity and resistance patterns in previously untreated versus relapsing ovarian cancer patients provides evidence that the development of resistance to even one drug may be associated with the acquisition of resistance to several classes of compounds (Table 1). For example, except for vinblastine and melphalan, *cis*-platinum, adriamycin, and bleomycin showed higher sensitivity rates against cancers from previously untreated patients than against those from relapsing patients. Furthermore, this acquisition of resistance to various classes of anticancer agents may occur despite different postulated mechanisms of drug action. Clearly, the development of resistance to the alkylating agent melphalan conveyed tumor resistance to the DNA-intercalating (e.g., adriamycin) [8] and -shearing (e.g., bleomycin) [13] agents. However, all three of these agents exert cytotoxicity by interacting with DNA. Resistance to these apparently diverse drugs could be due to enhanced DNA-repair mechanisms in resistant TCFUs, although other mechanisms might also

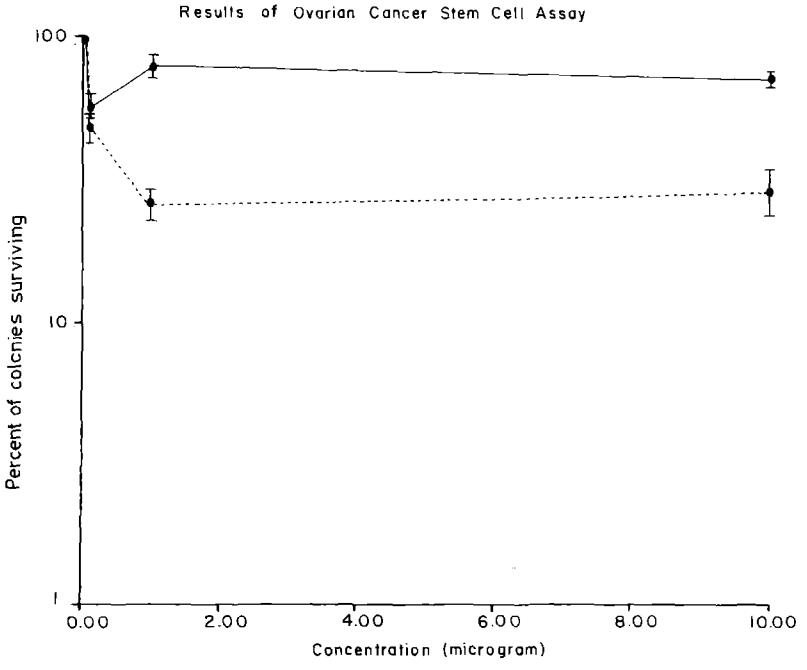

Fig. 2. The effect of bleomycin and vinblastine on the inhibition of ovarian TCFUs from patient 2 (Table 5) in July, 1977. Note the lack of bleomycin activity, resulting in a flat dose-survival curve (●———●). (———) Bleomycin; (- - - -) Vinblastine

apply. Clinical reports have also shown that adriamycin is ineffective as a second-line therapy for ovarian cancer patients previously exposed to alkylating agents [7, 12, 21]. These in vitro and in vivo findings suggest that both adriamycin and bleomycin should be used as components of first-line therapy, but are unlikely to be useful for the treatment of relapsing patients. Similar studies of cross sensitivity and resistance patterns in other solid tumor types may be used to identify those drugs which may be most effective in both previously untreated and relapsing patient populations.

Although these drug sensitivity data were obtained through in vitro drug studies on TCFUs from 115 patients, a clinical trial yielding similar data would have required that nearly 450 evaluable ovarian cancer patients be treated with single agents. In addition to revealing important cross resistance relationships between different classes of anticancer agents, these in vitro studies have identified vinblastine, bleomycin, and mAMSA as potentially useful agents in the therapy of ovarian cancer patients. Vinblastine had similar in vitro activity to *cis*-platinum and was not cross-resistant with it. Bleomycin had little activity against TCFUs from patients who had received prior alkylating agents, but was an effective drug in previously untreated patients. mAMSA appears to be less cross-resistant and may prove clinically useful in the setting of adriamycin resistance. Finally, preliminary data on TCFUs from ten ovarian cancer patients showed that vindesine and vinblastine were completely cross-resistant with one another. At least for ovarian cancer it is unlikely that vindesine will prove more useful than vinblastine [15].

Melphalan had less activity in vitro than has been reported for it in clinical trials as primary treatment for ovarian cancer (i.e., 13% in vitro versus 25% in vivo sensitivity rates) [6, 26, 27], but the confidence limits for in vitro sensitivity of 0–33% encompass its clinical response rate. While the in vitro result may be valid it is also possible that our in vitro incubation medium (i.e., McCoy's 5A), which contains relatively high concentrations of leucine and glutamine, may have blocked the uptake of melphalan into the ovarian TCFUs and thus resulted in a falsely low in vitro sensitivity rate [23, 24]. Future in vitro melphalan studies should evaluate the culture media, which contain concentrations of these amino acids that are no higher than would be found in the circulating plasma.

All the clinical correlative trials were carried out in ovarian cancer patients who had relapsed following treatment with an alkylating agent or with multiple-drug regimens containing adriamycin, cyclophosphamide, and/or *cis*-platinum. Clinical response rates to empirical second-line single-agent therapy in such patients are very low, averaging 0–25% in large series [12, 21, 28]. In view of these data the HTSCA's predictive accuracy of 73% for objective tumor response was considered quite good. It is important to point out that some patients included in this trial were entered according to a prospective correlative design [18] (rather than randomization). With this design, clinical trials of a single agent are carried out independently and generally simultaneously with laboratory testing. However, when assay results permitted selection of an agent to which the patient's TCFUs were sensitive, this agent was then prospectively selected in a decision-aiding mode [18] in relation to the clinical trial (Table 6). Unfortunately, only 11 of the 44 in vitro assays (25%) detected tumor sensitivity to a single drug, which could then be used in a decision-aiding clinical trial. As might be anticipated, as the number of drugs tested in vitro increases the percentage of patients sensitive in vitro to at least one drug also increases [17]. Over 78% of all patients whose tumors are successfully grown in vitro were sensitive to at least one of four or more drugs tested in vitro. When eight or more different drugs were tested in vitro, TCFUs from almost all patients manifest sensitivity to at least one of the drugs.

The extremely high true-negative rate (100%) accuracy of the in vitro assay in predicting clinical drug resistance in patients with advanced ovarian cancer clearly indicates that this assay can be used to exclude anticancer drugs which will not be clinically useful for tumor response but which could cause toxicity. The HTSCA for advanced ovarian cancer appears to have a similar predictive accuracy rate to that of the estrogen receptor assay for predicting the response to hormonal therapy for disseminated breast cancer [14].

Table 6. Stages of development in clinical trials with the tumor stem cell assay

1. Retrospective correlative trials – Clinical therapy and the in vitro testing are carried out independently
2. Prospective correlative trials – The clinical trial design dictates which specific agent or agents all patients are to receive clinically and to have tested in vitro
3. Prospective decision-aiding trials – The results of testing a large battery of drugs in vitro leads to the selection of a single agent or drug combination for clinical trial

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Unfortunately, the partial remissions in our patients were relatively short in duration (i.e., median of 3.3 months), reflecting the poor results attainable with currently available agents for second-line therapy even when drug sensitivity is predicted. Future studies based on the HTSCA may have greater value for the prediction of useful drug combination therapy for patients who have not previously received chemotherapy. Large prospective studies will be useful to determine the ultimate impact of culture and sensitivity selection of treatment on the complete remission rate and overall survival of patients with advanced ovarian cancer. The ultimate result of such prospective studies may lead to major changes in the design of future clinical trials and in the treatment of cancer patients.

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