The Value of the Human Tumor Cloning Assay in Ovarian Cancer

Axel-R. Hanauske, MD, and Daniel D. Von Hoff, MD

University of Texas Health Science Center at San Antonio
San Antonio, Texas

Ovarian cancer ranks among the most common causes of death from malignant diseases and is the leading cause of gynecologic cancer deaths in the United States. The number of deaths for 1984 has been estimated to be higher than 11,000, more than the estimates for deaths due to endometrial and cervical cancer combined.\(^1\) Also for 1984, approximately 17,000 new cases have been diagnosed.\(^2\) The incidence of ovarian cancer in women ages 35 or older lies between 10 and 32 per 100,000 and has increased over the last decade.\(^3\) Although considerable progress has been made in the diagnosis and therapy of the disease, 5-year survival rates are still in the range of only 30–40%.

The clinical course of ovarian cancer is characterized by the confinement of the disease to the abdominal cavity over a long period of time and a scarcity of distant metastases. Often, local complications are the ultimate cause of death. Systemic and regional chemotherapy are important in managing advanced ovarian cancer. At present the choice of drugs is made empirically on the basis of clinical studies or the individual experience of the clinical oncologist. It would be more rational, however, and probably save the patient inactive regimens with toxic side effects, if there were a way to determine the sensitivity or resistance of an individual patient's tumor to antineoplastic agents with the use of some type of predictive test.

In this article we will summarize the potentials and limitations of one type of predictive tests, the human tumor cloning assay (HTCA), in the management of ovarian cancer.

The Stem Cell Hypothesis

The basis for any clinical potential of the HTCA is provided by the stem cell hypothesis for human malignancies.\(^4\) Basically, it suggests that all cells within a solid neo-
plasm can be assigned to one of the following categories:

1. Stem cells, which retain the ability for unlimited self-renewal. Some daughter cells become transient cells.
2. Transient cells enter tissue-specific differentiation steps and can undergo only a limited number of divisions.
3. End-stage-differentiated cells with no ability for further cell divisions

Because the stem cell compartment eventually produces all other tumor cells, identification and destruction of these cells are of utmost importance for the treatment of malignant diseases.

The Human Tumor Cloning Assay

The HTCA, although probably not entirely specific for stem cells,\textsuperscript{5,6} accomplishes the selection of growing neoplastic cells by use of a semisolid medium.\textsuperscript{7,8} The principal steps are outlined in Figure 1. Briefly, starting from solid tumor specimens or malignant effusions in advanced ovarian cancer, a single-cell suspension must be prepared. The cells then are exposed to antineoplastic agents for 1 hour. After the drugs are removed by repeated washings, agar is added to the cell suspension (final concentration: 0.3%) and the mixture is transferred to petri dishes containing a base layer of 0.5% agar. With some drugs a continuous exposure is needed and is accomplished by leaving the drug in the cell–agar mixture. The most important function of the agar base layer is to prevent contact of the cells with the plastic dish. This would promote growth of nonmalignant cells, mainly fibroblasts. The petri dishes then are incubated at 5% CO\textsubscript{2}, 37°C, 100% humidity for 14–21 days. Over this period some malignant cells multiply and eventually form colonies (Fig. 2).

Much work has been directed to prove that the colonies formed in the HTCA are indeed composed of malignant cells and are representative of the original tumor. In pioneering studies, Hamburger and Salmon\textsuperscript{7,9} showed by use of light microscopy and cytogenetic analysis the malignant nature of colonies from patients with ovarian cancer. Later, results of studies using nude mice demonstrated that pooled colonies from ovarian cancer specimens are capable of generating tumors in vivo.\textsuperscript{10} Trent and Salmon\textsuperscript{11} reported a variety of chromosome changes in cells from the colonies frequently including the partial deletion of the long arm of chromosome 6 and alterations of chromosome 1.

Assay results usually are evaluated with an inverted microscope. Two types of controls are necessary to guarantee reliable re-
HUMAN TUMOR CLONING ASSAY

The first is the solvent of the drugs to be tested (negative control). The second uses a highly cytotoxic agent to reliably kill all cells (positive control). It is used to avoid spurious high colony counts due to a poor single-cell suspension and the presence of cell clumps. Evaluability of an experiment is operationally defined as \( \geq 20 \) colonies in the negative control and the positive control having \( \leq 30\% \) colonies as compared with the negative control. Some investigations prefer \( \geq 30 \) colonies in the negative control to consider an experiment evaluable. Drug effects are expressed in percent survival of tumor colonies as compared with the negative control. Drugs are arbitrarily considered active if they lead to a reduction in the number of colonies of \( \geq 70\% \) as compared with the negative control.\(^{15}\)

In Vitro Growth of Ovarian Cancer Cells

Many investigators have evaluated the growth of ovarian cancer colonies in the HTCA. Table 1 summarizes the results. Findings regarding colony growth in the HTCA vary considerably among different investigators. This results in part from a lack of standardization of the technique. Also, the definition of a colony is operational and varies between investigators. Finally, as shown in Table 1, the lower the investigators set their definition for evaluable growth, the higher is the percentage of evaluable specimens. However, as we have already pointed out, a minimum of 20 colonies per 500,000 nucleated cells seeded presently is believed to be essential for drug testing in the HTCA. Using this criterion, evaluable growth can be achieved in 30–65\% of tumor specimens by most investigators. Only Welander et al.\(^{14}\) have reported growth in \( > 90\% \) of their specimens.

In a recent analysis of our data base, we found growth of \( \geq 20 \) colonies/500,000 nucleated cells in 52\% of specimens plated and growth of \( \geq 30 \) colonies/500,000 cells in 45\% of the specimens (\( N = 1,319 \)). Table 2 shows a comparison of in vitro growth from effusions and solid specimens. It demonstrates that a higher percentage of fluid than solid specimens form \( \geq 20 \) colonies/500,000 cells (\( \chi^2 = 22.8, \ P < 0.00001 \)).

Ovarian cancer colonies also can be grown from malignant peritoneal lavages in patients without ascites.\(^{15}\)

Clinical Correlations of HTCA Results in Ovarian Cancer

In large studies on heterogenous patient populations, the HTCA predicted in vivo (patient) tumor sensitivity correctly in

<table>
<thead>
<tr>
<th>Authors</th>
<th>No. Specimens Attempted</th>
<th>Percent Evaluable Growth</th>
<th>Definition of Evaluability (No. colonies/10^6 cells seeded)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schlag and Flentje(^{33})</td>
<td>7</td>
<td>71</td>
<td>( \geq 5 )</td>
</tr>
<tr>
<td>Hamburger and Salmon(^{18})</td>
<td>8</td>
<td>100</td>
<td>Data not available</td>
</tr>
<tr>
<td>Ozols et al.(^{15})</td>
<td>20</td>
<td>75</td>
<td>( \geq 5 )</td>
</tr>
<tr>
<td>Ozols et al.(^{34})</td>
<td>27</td>
<td>81</td>
<td>( \geq 5 )</td>
</tr>
<tr>
<td>Hamburger et al.(^{9})</td>
<td>31</td>
<td>85</td>
<td>( \geq 1 )</td>
</tr>
<tr>
<td>Rey et al.(^{32})</td>
<td>50</td>
<td>30</td>
<td>( \geq 30 )</td>
</tr>
<tr>
<td>Natale and Kushner(^{17})</td>
<td>52</td>
<td>65</td>
<td>( \geq 30 )</td>
</tr>
<tr>
<td>Von Hoff et al.(^{19})</td>
<td>71</td>
<td>75</td>
<td>( \geq 5 )</td>
</tr>
<tr>
<td>Silkie et al.(^{16})</td>
<td>82</td>
<td>64</td>
<td>( \geq 50 )</td>
</tr>
<tr>
<td>Welander et al.(^{14})</td>
<td>113</td>
<td>91</td>
<td>( \geq 30 )</td>
</tr>
<tr>
<td>Williams et al.(^{12})</td>
<td>138</td>
<td>43</td>
<td>( \geq 30 )</td>
</tr>
<tr>
<td>Von Hoff(^{32})</td>
<td>411</td>
<td>60</td>
<td>( \geq 20 )</td>
</tr>
</tbody>
</table>
TABLE 2. Comparison of In Vitro Growth of Malignant Effusions Versus Solid Specimens in Ovarian Cancer

<table>
<thead>
<tr>
<th></th>
<th>Effusions</th>
<th>Solid Specimens</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. received</td>
<td>655</td>
<td>738</td>
<td>1,393</td>
</tr>
<tr>
<td>No. plated*</td>
<td>621</td>
<td>698</td>
<td>1,319</td>
</tr>
<tr>
<td>Growth†</td>
<td>362</td>
<td>315</td>
<td>677</td>
</tr>
<tr>
<td>Percent growth†</td>
<td>58</td>
<td>45</td>
<td>51</td>
</tr>
</tbody>
</table>

The percentage of tumors forming colonies from malignant effusions is significantly higher than from solid specimens (χ² = 22.8, P = 1.8 × 10⁻⁶). *Reasons for not plating a specimen were lack of cells or obvious bacterial contamination. †Greater than or equal to 20 colonies/500,000 cells seeded.

60–88% and in vivo (patient) tumor resistance in 85–94%¹⁰⁻²¹ of specimens tested. Similar results have been obtained for ovarian cancer. In an early study on 20 patients with 53 retrospective correlations, Salmon et al.²² reported 60% true sensitive and 100% true resistant predictions by the assay. A prospective study in 40 patients with ovarian cancer confirmed these results.²³ Currently, Welander et al.²⁴ are performing a trial in patients with advanced ovarian cancer who have not received prior chemotherapy. The authors compare treatment with the three drugs that were most active in the HTCA with standard cyclophosphamide, adriamycin, cisplatin (CAP) therapy. In an interim report,²⁵ the authors pointed out that, even if no drug led to a significant cell kill in the HTCA, the “best-three-drug” therapy led to the same clinical response rate as CAP therapy. The response rate in the CAP-treated group of patients was 12% (3 of 25 patients). The response rate in the best-three-drug group was 12.5% (3 of 24 patients). The probability for a clinical response was higher when more drugs were active in the HTCA (≥ 50% colony survival). Seventeen of 20 patients with in vitro–sensitive tumors responded to the best-three-drug therapy (85%), while 17 of 25 patients treated with CAP had a response (65%). The difference was not significant (P = 0.17). However, HTCA-guided chemotherapy led to a higher response rate than CAP therapy in patients with performance status 2 and a residual tumor mass of > 1 cm (10 of 10 patients versus 5 of 9 patients, P = 0.033). Welander et al. are continuing this study to determine whether these findings hold true in a larger group of patients.

Alberts et al.²⁶ studied 69 patients with relapsed ovarian cancer and assigned the patients to one of the following groups:

1. Tumor sensitive to one or more drugs in the HTCA, patient treated according to in vitro results
2. tumor sensitive to one or more drugs in the HTCA, patient treated with clinician’s choice of drug
3. tumor resistant in the HTCA

The assignment of patients to groups 1 and 2 was not randomized but left to the referring physician’s judgment. Treatment was with single agents or combination therapy. Clinical response rates were 54% (group 1), 20% (group 2), and 8% (group 3). The differences were statistically significant.

Hug et al.²⁷ have reported that in vitro growth for stage III and stage IV ovarian cancer of tumor colonies, per se, does not select treatment responders.

In summary, the predictive power of the HTCA for patient response in advanced ovarian cancer is in good agreement with data generated from larger heterogeneous populations. It seems that HTCA-guided chemotherapy is superior to empirically chosen regimens. However, at present, sample sizes are small, ongoing studies are not finished, and most of the clinical responses are only partial responses (> 50% decrease in tumor size but not complete disappearance of disease). Most important, the most powerful and reliable clinical trial design, the randomized and stratified trial, has not been performed for single-
agent therapy. Welander recently introduced randomization and stratification procedures into his study of the three-best-drug treatment.\textsuperscript{24} We hope the outcome of this important trial will provide crucial information on the benefits of the HTCA in the clinical setting.

**The HTCA and Patient Survival**

Another crucial question for the physician involved in the management of ovarian cancer patients is whether any in vitro method can help prolong patient survival. Two variables of the HTCA can serve as end points for survival studies: 1) the ability of tumor cells to grow in vitro as measured by the cloning efficiency (CE; number of colonies/number of cells plated), and 2) the drug sensitivity pattern of tumor colonies in the HTCA with or without according treatment of the patient.

**Patient Survival and Colony Formation in the HTCA**

Bertoncello et al.\textsuperscript{28} and Rey et al.\textsuperscript{29} have independently studied the relationship of CE and patient survival. Rey et al. found that CEs of $<6 \times 10^{-5}$ correlated with patient survival of $>12$ months and CEs of $>6 \times 10^{-4}$ correlated with survival of $<2$ months. In the study of Bertoncello et al.,\textsuperscript{28} four patients with CEs of $\geq 3 \times 10^{-3}$ survived for $74 \pm 45$ days, while five patients with CEs of $\leq 6 \times 10^{-4}$ survived for $103 \pm 39$ days. A survival of $203 \pm 42$ days was noted in eight patient whose tumors did not grow in the HTCA.

In summary, available studies, although scarce, indicate that high CEs correlate with poor patient prognosis in patients with ovarian cancer.

**Patient Survival and Drug Sensitivity Pattern in the HTCA**

Alberts et al.\textsuperscript{30} have studied 54 patients with relapsed ovarian cancer. The patients were assigned to one of the following groups:

- A. Tumor sensitive in the HTCA and patient treated according to assay results ($n = 17$)
- B. Tumor sensitive in the HTCA but patient treated empirically ($n = 20$)
- C. Tumor resistant in the HTCA and patient treated empirically ($n = 17$)

The prior chemotherapy status of patients in all groups was comparable. The median survival of patients treated according to HTCA results (group A) was 10.5 months. However, patients in both control groups (groups B and C) survived for a median of only 3 months ($P < 0.005$).

In another study on 69 patients with relapsed ovarian cancer,\textsuperscript{26} Alberts et al. formed the same groups. The survival curves were comparable for the first 3 months after treatment. Thereafter, they split and the authors concluded that HTCA-guided chemotherapy leads to an increase in actuarial survival. In an additional approach, the authors regrouped their patients according to HTCA results.

1. Tumor resistant to all drugs tested in the HTCA
2. Tumor sensitive to one drug only in the HTCA
3. Tumor sensitive to more than one drug in the HTCA

The median survival rate did not differ between the three groups. These data suggest that patients with in vitro sensitive tumors have no longer life expectancy than patients with resistant tumors unless they are treated according to HTCA results.

In conclusion, although available data are incomplete, HTCA-guided chemotherapy might lead to prolonged survival of patients with ovarian cancer.

**The HTCA and Drug Development in Ovarian Cancer**

The HTCA not only offers potential advantages for the clinical management of patients, but also for the development and preclinical testing of prospective new anti-
neoplastic agents. More than 10,000 new chemical compounds now are screened by the National Cancer Institute each year for antitumor activity. A mouse leukemia (P 388) serves as a first-line test system, and only compounds active against this leukemia are tested further for antitumor activity in mouse tumors and human tumor xenografts. This approach is advantageous in dealing with the myriad of chemicals, but it has several pitfalls. Chemicals that are inactive against the P 388 mouse leukemia might be active against human solid neoplasms.\textsuperscript{31} The place of the HTCA in the rational design of drug development has been the subject of many publications. Recently, a study has been published that combined the efforts of four major groups of investigators and was coordinated by the National Cancer Institute.\textsuperscript{31} The report provides important recommendations for quality control measurements for the use of the HTCA in drug development. Out of 79 drugs tested in the HTCA that were inactive against the P 388 leukemia, 14 had considerable antitumor activity in the HTCA. The results provided evidence that the HTCA can be used successfully in the search for new anticancer drugs if appropriate controls are used.

**Final Conclusions**

The HTCA is a versatile in vitro system for studying fresh human tumor specimens. There is ample evidence that the colonies formed from ovarian cancer specimens are indeed composed of malignant cells. Studies of the clinical importance of assay results are ongoing, and available data suggest that HTCA-guided therapy might be superior to the empirical choice of chemotherapy. Data on patient survival are scarce but indicate a survival advantage for patients whose chemotherapy is selected by the HTCA, although it is not as dramatic an advantage as one would like to see. In vitro colony formation, per se, seems to be a prognostic variable in ovarian cancer.

At present, however, there are serious limitations to the routine application of the HTCA:

1. The assay still has methodologic difficulties. Not all tumors grow in the assay. Although ovarian cancer belongs to the best-growing tumor types, often 30–50% of specimens are lost. The reasons for this are complex and involve damage during the preparation of the single-cell suspension as well as disturbance of the microenvironment of the cells. The optimal growth conditions for the tumor cells have yet not been determined, and much further work is needed. The preparation of true single-cell suspensions is tedious and sometimes is not successful. Enzymatic treatment of cells often is used but might harm membrane structures. The concern that enzymes might alter the drug sensitivity pattern of tumor cells in the HTCA is still valid. Many questions remain unanswered as to drug stability under assay conditions, optimal duration of drug exposure, and optimal drug concentration for in vitro/in vivo correlations.

2. At present, no randomized and stratified clinical trial has demonstrated the superiority of HTCA-guided single-agent therapy over the empirical clinician's choice of drugs.

3. Studies on the impact of HTCA parameters (growth, drug sensitivity pattern) on patient survival are encouraging but preliminary. As pointed out, randomized trials are needed.

When all factors are considered, we cannot recommend the routine clinical use of the HTCA because it still must be considered an experimental procedure. However, we would like to encourage efforts to make ovarian cancer specimens available to centers with ongoing studies designed to answer these important clinical questions. More randomized studies with stratification for other confirmed or possible prognostic factors (performance status, tumor grade, stage of disease, residual tumor mass after debulking, etc.) need to be performed before the true value of the HTCA in patient care can be determined.
References


