Human Ovarian Carcinoma Cells Maintained on Extracellular Matrix versus Plastic

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ABSTRACT

The ability of culture dishes coated with an extracellular matrix (ECM) to act as a suitable substrate for human ovarian carcinoma cells in vitro has been examined. The plating efficiency on ECM was 30 to 80% (dispersed tumor cells from solid tumor tissue and effusions) with active proliferation of tumor cells being observed. Within a few days, ovarian carcinoma cells seeded on an ECM were noted to attach firmly and assume a flattened morphology. In addition, ovarian carcinoma cells maintained on ECM-coated dishes could be released easily via trypsinization or with a cell scraper. This is in marked contrast to tumor cells seeded onto plastic dishes without an ECM. Invasion through the ECM by tumor cells from solid tumor tissue was occasionally noted.

Nonmalignant cells were removed from dispersed tumor cell preparations by preplating on plastic culture dishes without an ECM. The malignant origin of the tumor cells was confirmed by morphological, histochemical, and cytogentic criteria.

This culture system represents a significant improvement over current methods for routinely culturing human ovarian carcinoma cells. Such a model may be utilized for screening anticancer drugs for their ability to inhibit proliferation of human ovarian carcinoma cells from individual patients. This system also may be useful for elucidating mechanisms of ovarian tumor cell attachment and invasion in the process of metastasis.

INTRODUCTION

In spite of recent advances in chemotherapeutic agents, ovarian carcinoma carries a poor prognosis for most patients. Because of the difficulty in maintaining human ovarian carcinoma cells under cell culture conditions, in vitro models for evaluating antitumor agents have not been extensively pursued. In vitro models reported recently have used soft agar culture systems (7, 14) and nude mouse systems (2). In the case of soft agar systems, the plating efficiency is quite low. Plating efficiencies vary from 0.001 to 1% at best (7, 16) with cell lysis generally occurring after 21 days (7). The colony-forming assay utilizing soft agar systems, the plating efficiency is quite low.

Using culture dishes coated with an ECM, the ECM has been demonstrated to serve as an adequate substrate upon which to culture a variety of anchorage-dependent human tumor cells that otherwise have been difficult to maintain in culture. Human prolactin-producing pituitary tumor cells (1), human hepatocarcinoma cells, and Ewing’s sarcoma cells (17) will firmly attach to ECM and form highly flattened and closely apposed epithelioid-like cells. In addition, the latter 2 cell types maintained on ECM had a higher growth rate as well as a lower serum requirement than those maintained on plastic culture dishes.

This report describes the growth of human ovarian carcinoma cells maintained on culture dishes coated with an ECM produced by bovine corneal endothelial cells. Because of the high plating efficiency and ability to support the proliferation of ovarian carcinoma cells, this system represents a significant improvement in the ability to routinely culture human ovarian carcinoma cells.

MATERIALS AND METHODS

FGF from bovine brain was prepared as described by Gospodarowicz et al. (3). The partially purified preparation of FGF obtained from the Sephadex G-75 chromatography was used in this work. It was found that the main peak of activity after gel filtration on Sephadex G-75 contained 10% of the activity of purified FGF on a weight basis (courtesy of Dr. D. Gospodarowicz, University of California at San Francisco). It was determined that 250 ng of partially purified FGF per ml (equivalent to 25 ng of purified FGF per ml) were a saturating concentration of FGF for the proliferation of bovine corneal endothelial cells used in the preparation of dishes coated with ECM.

Fetal bovine serum, calf serum, RPMI 1640, and DMEM were purchased from Grand Island Biological Co. (Grand Island, N.Y.).

Cell Dispersion. Solid tissue from primary or metastatic ovarian carcinoma was processed within 1 hr after it had been surgically excised.4 Areas of fat, nontumor, or obviously necrotic tissue were discarded. Viability of solid tumor tissue was confirmed with a scalpel into approximately 2- x 2- x 2-mm pieces. The minced tissue was placed in a Petri dish containing RPMI 1640 supplemented with 1% kanamycin for approximately 2- x 2- x 2-mm pieces. The minced tissue was placed in a Petri dish containing RPMI 1640 supplemented with 1% kanamycin for 30 min at 37° (95% O2:5% CO2). The ovarian carcinoma tissue was then incubated in RPMI 1640 containing 4 mg collagenase per ml (type III; Millipore Corp., Bedford, Mass.) and 0.1 mg bovine pancreatic DNase per ml (Sigma Chemical Co., St. Louis, Mo.) for 1 hr at 37° (95% O2:5% CO2). The ovarian carcinoma tissue was then centrifuged at 500 x g for 10 min and resuspended in 2 ml of isotonic Earle’s salt buffer and then handled similarly to the dispersed cells in soft agar.

Ovarian carcinoma cells in effusions were removed by centrifugation at 500 x g for 10 min. The cells were then resuspended in 2 ml of isotonic Earle’s salt buffer to be placed on a preformed gradient of Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.).

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were mixed with one part 10-fold-concentrated Earle's balanced salt solution containing 0.7% bovine serum albumin (Sigma) and 250 mM N'-2-hydroxyethylpiperazine-N'-ethanesulfonic acid. The Percoll solution was diluted with isotonic Earle's salt buffer containing 0.07% albumin to give a 45% Percoll solution, pH 7.4. Twelve ml of the 45% Percoll solution was placed in an 15-ml polystyrene tube and centrifuged for 1 hr at 29,000 x g in a 34° angle rotor (Sorval Model RC2). Dispersed ovarian carcinoma cells in 2 ml of isotonic Earle's salt buffer were layered carefully on top of the gradient and centrifuged at 800 x g for 20 min at 20°. Density marker beads suspended in 2 ml of isotonic Earle's salt buffer were layered carefully on top of a reference gradient to determine the density and shape of the gradient. The bottom of the centrifuge tube was pierced with a tube-piercing needle, and 0.5-ml fractions were collected. Morphology of the cells was analyzed using a phase-contrast microscope, and the cell number was determined with a hemocytometer.

The 0.5-ml fractions were diluted with 4.5 ml of RPMI 1640 and centrifuged for 20 min at 800 x g. Cell pellets were resuspended in RPMI 1640 containing 20% fetal bovine serum. The cells were pooled according to individual cell fractions. RBC and debris could easily be separated from fractions containing tumor cells and be discarded.

**Cell Culture Conditions.** Prior to plating, the ovarian carcinoma cells were placed in a 10-cm plastic culture dish (without an ECM) of 10 ml of RPMI 1640 supplemented with 20% fetal bovine serum for 1 to 2 hr. Fibroblasts and mesothelial cells tended to attach to the plastic substrate during this period, leaving a relatively pure preparation of tumor cells in suspension. The tumor cells could be preplated on plastic substrate with subsequent passages to eliminate any fibroblasts present. The suspended tumor cells were centrifuged at 500 x g for 10 min and resuspended in complete medium. The tumor cells were maintained on culture dishes (with or without an ECM) in the presence of RPMI 1640 supplemented with 20% fetal bovine serum, Fungizone, and gentamicin.

**Culture of Bovine Corneal Endothelial Cells.** Primary culture of bovine corneal endothelial cells was done as described by Gospodarowicz et al. (6). The corneas from healthy steer eyes were carefully excised avoiding the iris. The posterior corneal surface was washed several times with phosphate-buffered saline (Ca²⁺ and Mg²⁺ free). Using a curved spatula, the posterior cornea was gently scraped while carefully avoiding the cut edges. Sheets of endothelial cells removed in this fashion were placed in 6-cm dishes supplemented with DMEM, H-16 (1 g glucose per liter), 10% fetal calf serum, 5% calf serum, glutamine, gentamicin (50 µg/ml), and Fungizone (2.5 µg/ml). The cultures were left undisturbed for 6 days, at which time, colonies of endothelial cells were present. The medium was renewed, and partially purified FGF (250 ng/ml) was added every other day. By Day 9, the cells were generally ready to be passaged to form solid plaques. Stock cultures were maintained in DMEM, H-16 supplemented with 10% fetal calf serum, 5% calf serum, gentamicin, and Fungizone. FGF (250 ng per ml) was added every other day until the cells were nearly confluent. Stock cultures were passaged every week at a split ratio of 1:64.

**Preparation of Plates Coated with an ECM.** Corneal endothelial cells from stock plates were dissociated with 0.5% trypsin-0.02% EDTA solution, and 10⁴ cells were seeded into each 35-mm dish. The cells were maintained in the presence of DMEM, H-16 supplemented with 10% fetal calf serum, 5% calf serum, 5% dextran T-40, gentamicin (50 µg/ml), and Fungizone (2.5 µg/ml). Partially purified FGF (250 ng/ml) was added every other day. Once the cultures became confluent (ordinarily within 6 days), media were renewed, and the cultures were further incubated for 6 days. The cultures were then washed with distilled water and exposed for 5 min to 0.02 M NH₄OH in distilled water. The nuclei and the ECM became visible, the cultures were washed 3 times with phosphate-buffered saline.

**Karyotyping of Cells.** Dispersed ovarian carcinoma cells in 10 ml of RPMI 1640 containing 15% fetal bovine serum and 1% kanamycin were placed in a 25-cm culture flask. Roughly 2 drops of a stock solution of Colcemid (0.2 µg/ml) were added to the suspension and incubated for 2 hr at 37° (95% O₂,5% CO₂). The cell suspension was then centrifuged for 10 min at 500 x g, and the supernatant was discarded. One to 2 ml of prewarmed 0.075 M KCl were added, and the suspension was gently mixed. Additional 0.075 M KCl was added slowly to bring the total volume to 10 ml. The cells were gently mixed with a Pasteur pipet. The cell suspension was incubated for 10 min at 37°, and then, 4 to 5 drops of fixative (glacial acetic acid:100% methyl alcohol, 1:3) were added. The suspension was gently mixed and centrifuged for 10 min at 500 x g. The supernatant was discarded, 1 to 2 ml of fixative were added, and the cells were resuspended. Additional fixative was slowly added to bring the total volume to 10 ml. The cells were gently mixed and centrifuged at 500 x g for 10 min. The above steps were repeated, beginning with the addition of 1 to 2 ml of fixative. When the final pellet was obtained, the cells were ready to be placed on glass slides for Giemsa staining or could be stored indefinitely in 10 ml of fixative at −15 to −20°.

For Giemsa staining, the cell pellet was suspended in 0.5 ml of fixative and dropped onto glass slides. The slides were gently flamed and air dried for several hr. The slides were then stained with a 3% Giemsa (Grand Island Biological Co.) solution for 12.5 min. For ovarian carcinoma cells maintained on ECM-coated dishes, Colcemid (0.2 µg/ml) was added directly to the culture dishes in RPMI 1640 supplemented with 20% fetal bovine serum for 2 hr at 37°. The cells were dissociated from the ECM with 0.5% trypsin:0.02% EDTA solution. The cell suspension was then centrifuged for 10 min at 500 x g, and the supernatant was discarded. One to 2 ml of 0.075 M KCl were added, and the procedure was continued as described above for dispersed ovarian carcinoma cells.

**Chromium Labeling of Tumor Cells.** Human ovarian carcinoma cells were incubated with 0.1 µCi of sodium ⁶¹Cr/chromate (Amersham/Searle Corp., Arlington Heights, Ill.) for 45 min at 37° (95% O₂,5% CO₂). The suspension was washed 3 times with RPMI 1640 supplemented with 20% fetal bovine serum. The cell suspension was further incubated 1 hr at 30° to allow leaching of ⁶¹Cr from the cells. The cell suspension was washed once with complete medium and then used for attachment studies. Cell viability before and after labeling with ⁶¹Cr was determined by the trypan blue exclusion method. At the indicated time points, the labeled tumor cells in the supernatant were washed once and counted. The labeled tumor cells attached to the culture dishes were released via trypsinization and counted. The attachment of tumor cells at any one time point was expressed as a percentage of radioactivity.

**Analysis of Data.** For the proliferation studies, the results are expressed as the mean cell number of triplicate plates (± S.D.) for each experiment. Statistical significance of the differences between groups was calculated using analysis of variance.

**RESULTS**

Specimens were obtained from 9 patients with ovarian carcinoma and one patient with endometrial carcinoma (Case 10) as noted in Table 1. Many of the patients had recurrent tumor and had been on chemotherapy in the past. The source of the tumor cells was from solid tumor tissue (300 mg to 2 g) and ascitic fluid (200 ml to 2 liters). When viable tumor cells were obtained, 100% of the samples were successfully cultured on ECM. In some cases, necrotic tumor tissue was received, and no viable tumor cells were noted after enzymatic dissociation. In one case, ascitic fluid grew only mesothelial cells, and cell blocks from the original ascitic fluid sample examined by the pathologist failed to reveal any malignant cells. In general, the number of viable tumor cells obtained from any one sample was 8 x 10⁶ cells or less.

**Cell Morphology.** The morphological appearance of the human ovarian carcinoma cells maintained on ECM-coated dishes varied considerably. The appearance varied from sheets of tumor cells forming a monolayer to heaped-up islands of tumor cells on top of a base of flattened, closely apposed tumor cells. If tumor cells formed islands of heaped-up cells, then those tumor cells farthest away from the ECM were noted to be less viable as
Table 1  
Histological and cytogenetic findings of the various cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Histological diagnosis</th>
<th>Tissue</th>
<th>Chromosome no. a</th>
<th>No. of cells examined</th>
</tr>
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<tr>
<td>1b</td>
<td>Poorly differentiated papillary</td>
<td>Ascites</td>
<td>43-145</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>adenocarcinoma ovary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Metastatic</td>
<td>36-105</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
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<td>Primary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Papillary adenocarcinoma, ovary</td>
<td>Primary</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>26-79</td>
<td>22</td>
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<td></td>
<td>adenocarcinoma ovary</td>
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<td></td>
<td></td>
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<td>34-105</td>
<td>8</td>
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<td>7</td>
<td>Borderline papillary serous adenocarcinoma, ovary</td>
<td>Primary</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>29</td>
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<tr>
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<tr>
<td>10</td>
<td>Well differentiated adenocarcinoma, endometrium</td>
<td>Primary</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Derived from analysis of tumor cells maintained on ECM.
  b Denotes prior chemotherapy administered to patient.

determined by the trypan blue exclusion method. Cells released into the medium from these islands could not be reseeded back onto an ECM. Fig. 1 demonstrates a primary culture of human ovarian carcinoma cells (Case 1) after 7 days on ECM. By this stage, proliferation of tumor cells has led to well-defined islands of tumor cells. Fig. 2 demonstrates a primary culture of the same tumor cells (Case 1) after 7 days in culture on plastic dishes without ECM. Most fields demonstrated occasional mesothelial cells with only rare individual or groups of tumor cells that had attached to the plastic and assumed a flattened morphology. The tumor cells in primary culture maintained on plastic could not be released easily and plated back onto plastic, whereas tumor cells maintained on an ECM could be passaged repeatedly. Tumor cells from Case 1, which had been maintained continuously on ECM for over 9 months with repeated passaging, continued to form heaped-up islands characteristic of the tumor cells in primary cultures.

The culture life span of human ovarian carcinoma cells in culture was generally 3 to 4 weeks. During that time, active proliferation of tumor cells was noted in all cases, and tumor cells could be passaged repeatedly back onto ECM. In some cases, proliferation was noted after 10 days in culture with both proliferation and lysis of cells being observed for the remainder of the culture life span. In Case 1, tumor cells had been in culture for over 9 months with repeated passaging. Preplating on plastic culture dishes without an ECM prior to plating on ECM resulted in the complete removal of nonmalignant cells after 2 passages.

The use of ECM-coated dishes greatly facilitated the obtaining of a suitable number of metaphases from 10^5 cells or less. As opposed to tumor cells maintained on plastic dishes without an ECM, tumor cells maintained on ECM-coated dishes could be easily released with trypsin. In individual cases where cytogenetic carcinoma cells to ECM, tumor cells from Case 1, which had been passaged continuously for over 9 months on ECM, were labeled with ^51Cr. Attachment to ECM versus plastic was determined over time as a function of radioactivity associated with attached and nonattached tumor cells (Chart 1). After 15 min, almost 50% of tumor cells had attached to ECM and had begun to assume a flattened morphology in some cases. The percentage of tumor cells attached increased to over 70% by 3 hr. Only 7% of tumor cells had attached to the plastic culture dishes without an ECM by 3 hr. The results were similar if cell number was used instead of percentage of radioactivity. However, in the presence of clumps of tumor cells, the use of ^51Cr may give a better representation of the overall attachment of tumor cells. Attempts to break up the clumps of tumor cells generally resulted in the lysis of cells. In addition, tumor cells not attached to plastic after 24 hr could be seeded back on to ECM-coated dishes with attachment of up to 50% of viable cells to the ECM being observed.

To correlate attachment with proliferation of tumor cells from Case 1, the proliferation of these cells was compared on ECM versus plastic in the presence or absence of FGF (500 ng/ml) added every other day for 9 days (Chart 2). After 9 days in culture, a statistically significant difference in cell number was noted for cells maintained on ECM as opposed to plastic dishes without an ECM. While further increases were noted with FGF, the differences were not significant. In addition, tumor cells from Case 1 were plated on soft agar (courtesy of Dr. Y. Rustum, Roswell Park Memorial Institute), and little or no growth of tumor cells was noted on soft agar after 3 weeks.

**Tumor Markers.** The cytogenetic studies (Table 1) were consistent with the malignant origin of the cells maintained on ECM. The use of ECM-coated dishes greatly facilitated the obtaining of a suitable number of metaphases from 10^5 cells or less. As opposed to tumor cells maintained on plastic dishes without an ECM, tumor cells maintained on ECM-coated dishes could be easily released with trypsin. In individual cases where cytogenetic...
studies were not performed, the malignant origin of the cells was confirmed by morphological and cytological criteria. Smears of dispersed tumor cells were stained with hematoxylin and eosin as well as with special stains (mucin, periodic acid-Schiff, etc.). In addition, tumor cells could be maintained on ECM-coated glass coverslips, stained, and mounted permanently on glass slides for cytological examination. In all cases examined, typical features of malignant cells were noted. From 90 to 100% of the nonmalignant cells could be removed from the tumor cells by preplating on plastic culture dishes.

**Invasion and Digestion of ECM by Tumor Cells.** In general, invasion and digestion of the ECM were not observed with tumor cells derived from ascitic fluid. In 2 cases of solid tissue-derived tumor cells, digestion of the ECM was noted after a few days in culture (Figs. 3 and 4). In one (Case 9), where tumor cells from ascitic fluid and solid tumor tissue were obtained together, digestion of the ECM was noted for tumor cells derived from the solid tumor tissue but not from the ascitic fluid. While 60% or more of the tumor cells from the ascitic fluid attached to the ECM (Case 9), minimal proliferation was observed over 21 days. This was reflected in the inability to obtain metaphases for cytogenetic analysis after repeated attempts (2- to 14-hr exposure to Colcemid). These cells appeared viable and could be passaged back onto ECM during the culture span of 21 days. The malignant origin of the tumor cells from the ascitic fluid was confirmed from cytological examination of the cells.

**DISCUSSION**

The ability of ECM-coated dishes to act as a suitable substrate for human ovarian carcinoma cells represents a major advance in the development of an in vitro model system. This system appears useful in (a) screening antitumor drugs for individual patients, (b) providing a model for attachment and metastatic spread, and (c) identifying subpopulations of tumor cells (from individual patients) which are more metastatic than others.

Because of the high plating efficiency and ability to support proliferation, ECM-coated dishes appear to be useful in screening individual patients with ovarian carcinoma for in vitro sensitivity to antitumor agents. Current studies are in progress to determine the predictive value of this culture system.

Malignant cells possess the ability to spread or invade into surrounding tissues and form metastases (10). The initial process involves attachment of the tumor cells to a suitable substrate. Kramer and Nicolson (10) have demonstrated that some tumor cells have the ability to attach to vascular endothelial cells, leading to the retraction of the endothelial cells and subsequent attachment to the underlying ECM. These steps are involved in the process of blood-borne tumor metastasis. While blood-borne metastasis can occur in patients with ovarian carcinoma, spread via lymphatics and via mesothelial seeding is more common. Attachment to ECM represents a complex series of interactions that play a significant role in metastatic spread (10). Fibronectin (9), laminin (4), glycosaminoglycan (8), and collagen (17) have been implicated in the attachment process. Evidence would suggest that multiple interactions with these components are necessary for attachment (11). These components and others are present in the ECM produced by bovine corneal endothelial cells in vivo and in vitro (5, 17). ECM-coated dishes should provide a model to investigate the factors involved in the attachment of ovarian carcinoma cells to ECM as well as to model to investigate agents capable of blocking the attachment of the tumor cells to ECM.

There is a growing body of evidence to suggest that most cancerous tumors contain subpopulations of cells with different properties (12). B16 melanoma sublines of high invasive potential have been demonstrated to solubilize the subendothelial cell matrix at higher rates compared to B16 melanoma sublines of low metastatic potential (11). That such subpopulations of tumor cells exist in individual patients with ovarian carcinoma is suggested by the observation in this report that tumor cells derived from solid tumor tissue but not ascitic fluid from an individual patient (Case 9) were capable of digesting the ECM. In addition, initial responses to chemotherapy in patients with advanced ovarian carcinoma are followed in most cases by the growth of resistant tumor cells within 12 to 18 months (15). Because of the high plating efficiency and ability to culture tumor cells derived from both solid tumor and ascitic fluid on ECM, this model system should prove useful in identifying subpopulations of tumor cells. Because human ovarian epithelium, fallopian tube epithelium, and endometrial cells develop from a similar celomic epithelium, tumors derived from these epithelial cells share similar characteristics (13). Preliminary evidence in our laboratory with endometrial carcinoma cells (Case 10) and fallopian tube carcinoma cells suggests that ECM-coated dishes can act as a suitable substrate for these tumor cells. Thus, ECM-coated dishes may provide an excellent model for investigating a variety of gynecological carcinomas of epithelial origin.

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**REFERENCES**


**ECM and Human Ovarian Carcinoma Cells**

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**Chart 2. Influence of substrate and FGF on the proliferation of human ovarian carcinoma cells.** Human ovarian carcinoma cells (passage 8, Case 1) were seeded on 3.5-cm plastic culture dishes with (i) or without (P) an ECM. FGF (500 ng/ml) was added every other day. The cells were trypsinized and counted on Day 9. CI, mean of triplicate cultures; bars, S.D. *, p < 0.001; **, p < 0.05.


Human Ovarian Carcinoma Cells Maintained on Extracellular Matrix  *versus* Plastic

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