Tissue Culture Studies on Pleural Effusions from Breast Carcinoma Patients

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SUMMARY

The general lack of success that has attended attempts to establish tumor cell lines from solid specimens of breast carcinoma prompted our use of pleural effusions for this purpose. This paper summarizes tissue culture studies on five sequential pleural effusions from each of two patients with breast carcinoma. The morphology of cells in fresh specimens and cultures was documented by light and electron microscopy. Tumor cells were abundant in the fresh fluid specimens and they showed a high degree of viability. Fibroblasts were generally absent. Tumor cells in the fresh fluids and in cultures were morphologically similar to tumor cells cultured from solid breast carcinoma tissue. Nonneoplastic cells were found to survive or proliferate for 1 to 6 months in culture. Two cell types that grew readily in one of our media (medium BTN) resembled either fibroblasts or mesothelial cells by light microscopy. Electron microscopic studies indicated that the fibroblast-like cells were structurally modified epithelial cells. Our results demonstrate that pleural effusions provide a fertile source of tumor cells from which tumor cell lines can be established.

INTRODUCTION

Numerous attempts to culture human breast carcinoma cells have been made over the last 20 years, with limited success (1, 2, 4, 7, 10). A primary cause of failure has been the presence of fibroblasts that occur in practically all breast tissue specimens. These ubiquitous cells proliferate rapidly and tend to smother the slower growing tumor cells. Other contaminating cell types are also present. The viability of the tumor cells is usually low and many cells are necrotic. In the few instances where cultures have been established, the tissue has usually contained zones of rapidly growing tumor cells with little connective tissue stroma.

To avoid the problem of fibroblast contamination in the initial material, we have used pleural effusions from patients with breast carcinoma. These specimens often contain tumor cells in large numbers, present as single cells or clusters. The cells show a high degree of viability (50 to 95%) as measured by the trypan blue dye exclusion test.

Fibroblasts are rare or absent, although other nonneoplastic cells are usually present and can interfere with attempts to obtain pure cultures of tumor cells. We have now studied over 40 pleural fluid specimens, some of them sequential aspirates from the same patient. The results presented here are representative of our experience. They are mainly based on findings in 10 specimens, 5 obtained from each of 2 patients at intervals of 1 to 2 months. The tumor cells observed and 2 of the main nonneoplastic cells (fibroblast-like and mesothelial-like) are described in detail.

MATERIALS AND METHODS

Pleural fluids were obtained aseptically in vacuum bottles by thoracentesis and treated as outlined in Chart 1. After trying out numerous media considered adequate in the establishment of other types of tumor lines, we derived our own combination of components believed essential. Two types of media were used with all cultures. In addition, modifications of these 2 media including minor changes in the amounts of serum, hormones, and carbohydrates were also used.

Media

Medium BTN. This rather complicated medium contains a mixture of 3 established formulae: 40% Leibovitz’s L-15; 20% Waymouth’s MB752/1; and 20% Ham’s F-10 or F-12. To this base is added 10 to 20% fetal bovine serum and various hormones: 10 μg insulin per ml (zinc-crystalline, Elanco Products Co., Indianapolis, Ind.); 5 μg cortisol per ml (Solu-Cortef, Upjohn, Dallas, Texas); and 4 μg dexamethasone phosphate per ml (Decadron, Merck, Sharp and Dohme, Arlington, Texas). More recently 15 μg glutathione per ml (Calbiochem, Dallas, Texas) and 0.2% lactose have been included. Antibiotics in the form of 100 μg disodium carbenicillin per ml (Pyopen, Beecham-Massengill, Dallas, Texas), and 50 μg gentamicin per ml (gentamicin reagent solution, Schering Diagnostics, Port Reading, N. J.) were used.

Medium CIG. This is a modification by Leibovitz of his L-15 medium. To the basic L-15 are added the hormones and glutathione mentioned above, 7 to 10% fetal bovine serum, and 7 to 10% of the centrifuged, filtered pleural exudate from the patient whose cells are to be cultured or from another patient. A modification of this medium,
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CIGB, also contains 7 to 10% beef amniotic fluid. (All prepared media, amniotic fluid and serum were obtained from Grand Island Biological Co., Santa Clara, Calif.) Antibiotics (gentamicin and Pyopen) were also used.

Procedures

After the red blood cells in the specimen had been hemolyzed, the remaining cells were concentrated and distributed into various glass and plastic T-flasks and Leighton tubes (see Chart 1). The Leighton tubes contained coverslips either of plain glass or sprayed with Teflon, and these were removed at frequent intervals for morphological studies. Cultures in the flasks were maintained by media changes and subcultured when necessary until they no longer survived or until they became established cell lines.

For light microscopy of fresh pleural fluid hemolysates, centrifuged cell blocks were fixed in 10% formalin, embedded in paraffin, and sections stained with hematoxylin and eosin. Smears of the centrifugate were also made, fixed in 95% ethanol, and stained by the Papanicolaou procedure. For electron microscopy, a portion of the hemolysate was mixed with an equal volume of 4% glutaraldehyde and fixed for 30 min. The specimen was then centrifuged to produce a pellet that was washed for a minimum of 12 hr in buffer, postfixed with osmic acid, and embedded in Epon. One-μm sections were stained with methylene blue for light microscopy, and thin sections were stained with uranyl acetate and lead citrate and studied using a Zeiss EM-9S electron microscope.

In the preparation and in situ embedding of tissue culture monolayers for electron microscopy, all fluid exchanges took place within the Falcon flask. Specific areas of the culture were preselected, photographed, and marked on the surface of the flask and then covered by an inverted embedding capsule during polymerization. The Falcon flask was then trimmed to the margins of the Epon block and the remainder of the flask was dissolved in xylene. The inverted embedding capsules were readily fractured from the surrounding Epon and the blocks were then trimmed so that each preselected area of interest was at the apex of a pyramid.

RESULTS

Five pleural fluid aspirates were obtained from each of 2 women patients, F and M. Both had a previous histological diagnosis of carcinoma of the breast and had received initial surgical therapy. Table 1 summarizes some of the data on these specimens. Patient F had received various chemotherapeutic agents throughout the period over which the specimens were obtained, while Patient M had received none. Patient F, on January 15, 1973, was not responding to 5-fluorouracil therapy and was almost moribund at the time she was admitted to the hospital. First 2500 ml of pleural fluid were withdrawn, and then Cytoxan was given to which she responded favorably. Subsequent pleural fluid aspirates from this patient were smaller in quantity and contained fewer viable cells.

Cell Types Present. Red blood cells were eliminated by hemolysis (see Chart 1). Identifiable cells at the time of initiation of the cultures included leukocytes and histiocytes. Tumor cells appeared singly or as clusters which floated in the medium and frequently did not settle on the surface of the culture flask for days or even weeks. A number of other cell types were also observed. These included slender, dark, dendritic-like cells and irregular large cells with pale ameba-like edges and cytoplasm that appeared clear except in the immediate vicinity of the nucleus.

Lymphocytes, when present, disappeared in 2 to 4 weeks, while the dendritic cells persisted for a longer time but did not appear to divide. The tumor cells usually multiplied for 4 to 8 weeks as adherent or floating clumps, or they formed spreading plaques on the surface of the flask. During that time the large irregular cells with clear cytoplasm, which did not appear to form clumps, slowly multiplied, became more rounded as they filled the flask, and in the presence of other proliferating cell types intermingled so that the different cell types were difficult to distinguish. After 2 to 4 months and as many as 15 subcultures at dilutions of 1:2 to 1:5, this mixture often still persisted.

Morphological Studies. A preliminary study was made on primary monolayer cultures of a solid explant of breast carcinoma that had been growing 8 days. Fig. 1 is a phase-contrast light micrograph of the growing cells, and their fine structure is illustrated in Figs. 2 through 4. The moderately high nuclear:cytoplasmic ratio and prominent nucleoli can be seen. Chromatin material is diffuse in the interphase nucleus. The cytoplasm contains lipid droplets and relatively abundant ribosome-bearing endoplasmic reticulum arranged as flattened cisternae that typically have a parallel, concentric distribution. Prominent desmosomes unite the cells (Fig. 4) and bundles of tonofilaments extend from them into the cytoplasm.

The morphology of the tumor cells in freshly hemolyzed pleural fluid specimens was then studied. Fig. 5 shows a section of an Epon-embedded hemolysate containing clusters of tumor cells of varying size and shape. Electron microscopy revealed that tumor cells (Fig. 6) displayed features similar to those observed in the monolayer of the solid tumor explants. The specimen contained occasional degenerating cells, but no alterations in the fine structure of the tumor cells could be attributed to the hemolyzing process.

The opportunity to examine the fine structure of tumor cells in a growing cell line resulted from successful establishment of the epithelial tumor cell line MDA-MB-157 from a pleural effusion obtained from a patient, W (11). The appearance of the growing culture as seen by phase microscopy is shown in Fig. 7. Fig. 8 is an electron micrograph of portions of 2 cells. The features are similar to those of the tumor cells in the fresh hemolyzed pleural fluid, although lipid droplets are absent. Free polyribosomal complexes are numerous, and occasional lysosomes are present.

In tissue cultures, spindle-shaped cells frequently appeared at the periphery of attached cell balls (Fig. 9), and their shape suggested that they were contaminating fibro-
Table 1

Cell types in sequential pleural effusions from 2 patients with breast carcinoma

<table>
<thead>
<tr>
<th>Sample</th>
<th>Date</th>
<th>Systemic treatment</th>
<th>Elapsed time before thoracentesis (days)</th>
<th>Pleural effusion (ml)</th>
<th>Cell viability (%)</th>
<th>Principal types of cells found*</th>
<th>Survival or multiplication in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9/27/72</td>
<td>Halotestin</td>
<td>19</td>
<td>1800</td>
<td>60</td>
<td>E</td>
<td>E; 2-3 wk</td>
</tr>
<tr>
<td>2</td>
<td>10/24/72</td>
<td>5-FU</td>
<td>15</td>
<td>1900</td>
<td>90</td>
<td>M, F</td>
<td>M, F; 6 mo.</td>
</tr>
<tr>
<td>3</td>
<td>12/5/72</td>
<td>5-FU</td>
<td>23</td>
<td>800</td>
<td>Not done</td>
<td>E</td>
<td>E; 3-4 wk</td>
</tr>
<tr>
<td>4</td>
<td>1/15/73</td>
<td>5-FU</td>
<td>26</td>
<td>2500</td>
<td>95</td>
<td>M, F</td>
<td>M, F; 8-10 wk</td>
</tr>
<tr>
<td>5</td>
<td>2/27/73</td>
<td>Cytoxan</td>
<td>12</td>
<td>1400</td>
<td>25</td>
<td>E</td>
<td>E; 2 wk</td>
</tr>
</tbody>
</table>

**Patient F**

**Patient M**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Date</th>
<th>Systemic treatment</th>
<th>Elapsed time before thoracentesis (days)</th>
<th>Pleural effusion (ml)</th>
<th>Cell viability (%)</th>
<th>Principal types of cells found*</th>
<th>Survival or multiplication in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/23/72</td>
<td></td>
<td>1550</td>
<td>99</td>
<td>E, R</td>
<td>E, R</td>
<td>E, R; 3-5 mo.</td>
</tr>
<tr>
<td>2</td>
<td>12/12/72</td>
<td></td>
<td>1050</td>
<td>75</td>
<td>M, F, D</td>
<td>M, F, D</td>
<td>M, F, D; 4-5 mo.</td>
</tr>
<tr>
<td>3</td>
<td>1/11/73</td>
<td></td>
<td>1000</td>
<td>95</td>
<td>E, R</td>
<td>E, R</td>
<td>E, R; 4-6 wk</td>
</tr>
<tr>
<td>4</td>
<td>2/12/73</td>
<td></td>
<td>950</td>
<td>70</td>
<td>M, F, D</td>
<td>M, F, D</td>
<td>M, F, D; 2-3 mo.</td>
</tr>
<tr>
<td>5</td>
<td>3/27/73</td>
<td></td>
<td>900</td>
<td>Not done</td>
<td>E, R</td>
<td>E, R</td>
<td>E, R; 3-4 mo.</td>
</tr>
</tbody>
</table>

* E, epithelial-like cells (presumably tumor cells); R, small round floating cells (presumably tumor cells); M, mesothelial-like cells; F, fibroblast-like cells; D, dendritic-like cells; 5-FU, 5-fluorouracil.

Fluid collected in 500 ml vacuum plasma bottles with heparin

- Fluid shaken and distributed into 50 ml conical centrifuge tubes and centrifuged 10 min at 1000 rpm
- Volume of pellet noted and if red cells present pellet is resuspended in 10 volumes H₂O for 60 sec to hemolyze
- Cellular suspension immediately made isotonic with 10 x MEM solution and adjusted with NaHCO₃ to pH 7.4
- Suspension centrifuged 10 min at 800 rpm
- Cell Pellet resuspended in small volume of growth medium
- Largest portion of suspension distributed in tissue culture flasks and Leighton tubes for culture studies
- Sample processed for electron microscopy and Papanicolaou smear
- Excess cells frozen in liquid nitrogen
- Pellet material fixed in formalin for histological study

* Chart 1. Preparation of pleural fluids for tissue culture. MEM, minimal essential medium.
blistas. Fig. 10 shows Epon-embedded cells from the culture in Fig. 9 by light microscopy. The cells have a distinctly granular cytoplasm and vary in size and shape. The fine structure of the cells is illustrated in Figs. 11 and 12. The granular appearance is the result of an accumulation of lysosomes. Cell membranes lie closely apposed, and occasional thickenings of the membranes suggest poorly defined desmosomes. Sparse, irregular microvillous projections are present at the free cell surfaces, and in Fig. 12, the arrangement of the endoplasmic reticulum can be seen toward the periphery of the cell; flattened cisternae of ribosome-bearing reticulum are arranged in parallel layers.

The possibility that mesothelial cells might proliferate in pleural fluid cultures has to be considered. Fig. 13 demonstrates the presence in fresh pleural fluid of relatively large cells with desmosomes and associated tonofilament bundles. Between desmosomes, the cell membranes separate to produce irregular intercellular spaces. Microvilli occur on the free cell surfaces. The cytoplasm is less electron dense than that of the tumor cells described above; it contains relatively few ribosomes and many slender filaments.

These are features of mesothelial cells, and similarities have been observed in a cell type that grows slowly but widely in cultures from pleural effusions which is illustrated in Fig. 14. The cells are oval or round, with a relatively low nuclear:cytoplasmic ratio and prominent nucleoli. Some features of the fine structure of these cells are shown in Figs. 15 and 16. The chromatin material is diffusely distributed unless the cell is dividing. The abundant cytoplasm contains scattered, elongated mitochondria, and many polyribosomal complexes. Cisternae of endoplasmic reticulum are small and infrequent, and they do not form parallel stacks. Between the plasma membranes of adjacent cells, cell contact specializations are present (Fig. 16), and tonofilament bundles form a feltwork throughout the peripheral cytoplasm.

**DISCUSSION**

Pleural fluids offer a number of advantages over solid tumor explants in attempts to establish tumor cell lines. One is the opportunity to obtain a series of sequential specimens from the same patient. The number and viability of the tumor cells may then be correlated with treatment. Another is the scarcity of fibroblasts. Some effusions from patients with breast carcinoma do not contain tumor cells, but usually they are numerous, and their viability is high when contrasted with that of tumor cells obtained from solid tumor explants.

Breast carcinoma cells in electron micrographs show features common to epithelial cells, including the presence of desmosomes and associated tonofilaments. Lipid droplets may be found within the cytoplasm. Cells from a short-term culture of a solid tumor explant (illustrated in Figs. 1 through 4) and a tumor cell line from a pleural effusion (11) show similar features (Figs. 7 and 8).

The heterogeneity of the cell population in many pleural effusions is well known to the diagnostic exfoliative cytologist, who must frequently distinguish between tumor cells, inflammatory cells, and mesothelial cells. The presence of different cell types in the fresh exudates could account for the variety of morphological forms which appear in cultures made from these specimens. In certain instances, some cells float in the medium for 2 or more weeks and can be mechanically separated, by pouring, from dendritic-like or mesothelial-like cells which adhere to the surface of the culture flask. In the case of Patient M, a pure line of these floating small cells was obtained after several such transfers and has now become established as a new breast tumor cell line. In other instances, a larger cell may prevail, proliferating slowly as scattered clumps that overgrow any contaminating cell types and ultimately, after 3 to 4 months and a number of subcultures, become the only cell type remaining. The kinds of media used also influence which cells predominate in the culture and their size and shape. The rich Medium BTN encourages the proliferation of fibroblast-like cells in cultures from solid breast tumors and pleural effusions. Medium CIG, on the other hand, seems to favor the dendritic-like cells, the large clear (mesothelial-like) cells, and tumor cells.

The presence of spindle-shaped cells in some cultures has been mentioned. These were thought to be fibroblasts because of their appearance in light microscopic preparations and the frequency with which fibroblasts occur in cultures from solid breast tumor tissue. However, fibroblasts are rarely observed in pleural effusions, and spindle-shaped cells migrating from the periphery of clumps of tumor cells could conceivably be modified epithelial cells. To investigate this possibility a selected area of a monolayer culture containing these cells was processed for electron microscopy. Fine structural features of carcinoma cells were found, indicating that these elongated cells are structurally modified epithelial cells (Figs. 11 and 12). The granular appearance of the cells in light micrographs was found to be due to the presence of numerous lysosomes within the cell cytoplasm (Figs. 9 and 10). Their presence might be an indication that the cells are not completely healthy.

In our cultures from most effusions we have observed several morphological variants of cells which cannot be identified by light microscopy. Fewer than one-half survive more than 4 months or multiply through a number of subcultures. At the present time we simply designate them according to their appearance in light microscopic preparations, as for example "dendritic cells." We are currently studying them in detail and will describe them in a future publication.

The frequent presence in pleural effusions of mesothelial cells suggests that they might proliferate under conditions of tissue culture as a nonneoplastic cell type that could be mistaken for tumor cells. We have attempted to examine this problem in the course of our morphological studies. Fig. 13 shows cells from a hemolyzed fluid with ultrastructural features, including irregular channels between desmosomes, that are similar to those described in normal mesothelium (3, 6), in hyperplastic mesothelium (8), and in tumors derived from mesothelial cells (9, 5).

The cell type illustrated in Figs. 14 to 16 first appears as isolated cells that rapidly settle on the surface of the flask.
These are relatively large cells, usually ovoid in shape, closely apposed to one another as they grow across the flask surface. Their fine structure differs from that of the tumor cells in our hemolysates and monolayers. The cytoplasm is particularly abundant and less electron dense. These cells are joined by desmosomes with tonofilament bundles, and the latter are probably responsible for the fact that the periphery of the growing cells appears paler (Fig. 14) than the perinuclear cytoplasm where the organelles are concentrated. Since the ultrastructure of cells may be modified during in vitro growth, it is conceivable that these "M" cells, as we presently refer to them, may indeed be mesothelial in origin. We hope that our further morphological studies on these and other specimens may clarify the nature of these cells and of the various other morphological types that occur in cultures of pleural fluids from breast carcinoma patients.

REFERENCES

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