An Immunocytochemical and Ultrastructural Study of Heterogeneity in the Human Breast Carcinoma Cell Line PMC42

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INTRODUCTION

While a number of human breast tumor cell lines have been described, with the possible exception of HS578T, where two morphological forms were observed (1), they are essentially monomorphic in monolayer culture. This monomorphic character is also a feature of their ultrastructure, where this has been studied (2–7). Even when grown within a three-dimensional matrix (8) or capillary culture system (9), one such cell line (MCF-7) exhibits only a restricted degree of variation in cellular morphology. Recent publications (10, 11), however, have described the establishment from a pleural effusion of a human breast tumor cell line (PMC42), which continues to exhibit considerable morphological heterogeneity after 46 passages over 5 yr and retains this characteristic pleomorphism after single cell cloning (10). Cultures of this cell line also produce cords of viable cells which detach from the culture flask, float free in the medium, and continue to grow.

Previous studies of putative human breast tumor cell lines (2, 12) have indicated that ultrastructural information may prove useful in assessing the cell of origin for these lines and confirm their epithelial nature in certain cases. We have therefore undertaken an ultrastructural study of this cell line maintained as monolayer cultures, as floating cords of cells, and grown on collagen gels.

Further confirmation of the breast epithelial nature of PMC42 was sought by means of an analysis of the staining patterns obtained by use of antikeratin antibodies and a series of monoclonal antibodies raised against human milk fat globule membrane, whose abilities to recognize subsets of normal breast epithelial cells may be observed in breast tumors and breast tumor cell lines (13–17). The objectives of the study were therefore (a) to confirm the breast epithelial nature of the cell line and (b) to provide ultrastructural evidence of the morphological heterogeneity implied by phase-contrast observations in order to provide a firm foundation for future studies of functional as well as morphological heterogeneity.

MATERIALS AND METHODS

A total of 30 separate cultures of PMC42, grown in varying conditions, has been studied.

Monolayer cultures of PMC42 at passages 18 to 21 were dispersed using trypsin:verseine solution as described previously (10). The cells were suspended in RPMI 1640 containing 10% fetal calf serum, insulin (0.6 μg/ml), hydrocortisone (1 μg/ml), 10⁻⁵ M α-thioglycerol, penicillin (50 μg/ml), and streptomycin (50 μg/ml) and were plated on Thermaxan covespans (Flow Laboratories, Irvine, Scotland) in the wells of 24-well culture trays (Nunc Plastics, Gibco Ltd., Paisley, Scotland) and into Petriperm dishes (Heraeus Ltd., Brentwood, U.K.). The morphology of the cells was checked and photographed using a Wild M40 inverted phase-contrast microscope, and cultures containing representative cell types were processed for electron microscopy.

Pellets of monolayer cells were obtained by trypsinization, followed by low-speed centrifugation (200 × g for 5 min), and pellets of floating cords of cells were obtained by harvesting the floating cells and centrifugation (200 × g for 5 min).

Cells were also grown on collagen gels prepared from rat tail collagen by dissolving 2 g of rat tail tendon in 300 ml of 1:1000 glacial acetic acid. Prior to use, the solution was neutralized with a 2:1 mixture of 10× Eagle’s basal medium and 0.34 N NaOH and poured into culture wells.

Immunofluorescence. Confluent cultures grown on glass coverslips were incubated on ice for 1 h with 200 μl of either a polyclonal antibody raised against human callus keratin as described previously (18) (a gift from Dr. M. J. Warburton) diluted 1:50 or monoclonal antibodies LICR-LON M3, M8, M18, and M24 grown as ascites and diluted 1:100. The M-series monoclonal antibodies were raised against human milk-fat globule membranes isolated from fresh human milk as described previously (16). After three 5-min phosphate-buffered saline washes, the cells were incubated for 1 h with fluorescein isothiocyanate-labeled goat anti-rabbit IgG or goat anti-mouse IgG (Nordic, Maidenhead, U.K.) diluted 1:20, washed with three 5-min phosphate-buffered saline treatments, and mounted in PVA (Polaron, Watford, U.K.). Control cultures were incubated either in normal rabbit serum diluted 1:50, in the absence of a first antibody, or with a monoclonal antibody of the same class, but lacking reactivity with human breast epithelium.

Electron Microscopy. Cultures were fixed by replacement of culture medium with 2% glutaraldehyde in 0.05 M phosphate buffer at room temperature for 1 h. They were postfixed in 1% osmium tetroxide in 0.05 M phosphate buffer for a minimum of 2 h (maximum, 16 h for cells on collagen gels). Both fixatives were buffered to pH 7.2 to 7.4, and the

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osmotic pressure was adjusted to 350 mOsmol by addition of sucrose. Samples were dehydrated with ethanol, embedded via propylene oxide in Epon:Araldite (19), and polymerized at 60°C for 48 h. Dehydration and embedding times were extended for cultures grown on collagen gels as described previously (20).

Thick (1 to 2 μm) sections were stained with 1% toluidine blue for light microscopy, and areas were selected for electron microscopy. Petriperm cultures were cut both parallel and vertical to the surface of the culture dish. Thin sections (70 to 90 nm) were cut with a diamond knife on a Reichert OMU4, stained with methanolic uranyl acetate and lead citrate (21), and examined in a Philips EM400.

RESULTS

Immunofluorescence

While monoclonal antibodies LICR-LON M3 and M24 reacted with small groups or individual cells in cultures of PMC42, LICR-LON M8 and M18 stained almost every cell. The staining varied in intensity, but cells which were negative were rare (Fig. 1, a to c). Fig. 1d shows the staining pattern obtained using an antikeratin antibody and shows a similar staining pattern to that observed with other breast tumor cell lines (data not shown). All control populations were negative.

Morphology and Ultrastructure

Similarities were observed in the phase-contrast appearance of cultures of PMC42 and cultures of normal human breast epithelium prepared as described previously (22) but grown under similar conditions of hormone stimulation (i.e., with addition of hydrocortisone and insulin) in which extensive proliferation of the normal cells occurs (Fig. 2). Furthermore, although a number of isolated, dead, or dying cells, as distinct from viable cords, appear to slough off from the monolayer into the supernatant in all passages.

Accurate correlation of phase-contrast appearance and ultrastructural characteristics was possible on the areas selected from Petriperm cultures. Areas where the majority of cells corresponded to one cell type as defined by their phase-contrast appearance were selected for electron microscopy.

The existence of gradations between some of the morphological cell types makes precise definitions difficult, but the following cell types have been identified and studied in regions where their phase-contrast morphology corresponded with previous descriptions. The phase-contrast morphology of the various cell types has been described in detail elsewhere (10), and only a brief description will be given here.

Type 1 Cells
Phase Contrast. These cells are flattened cells of varying shapes with a clear cytoplasm (Fig. 3, inset) and devoid of vacuoles or any other distinguishing features. Most of the cell divisions that were observed were seen in areas corresponding to this cell type.

Electron Microscopy. Pale, rather small undifferentiated cells with relatively few cytoplasmic organelles were seen by electron microscopy in areas of cultures corresponding to type 1 as defined above (Fig. 3). Nuclei are irregular with prominent nucleoli. Where the cells are exposed to the medium, they have relatively few microvilli on their exposed membranes and are joined by occasional junctional complexes. Mitoses were observed relatively frequently in such cells.

Type 2 Cells
Phase Contrast. These cells are small cuboidal cells which grow densely, often forming "islands" in areas of other cells (Fig. 4a).

Electron Microscopy. The cells are cuboidal with distinctive polarization of organelles (Fig. 4a). The apical surface has large numbers of microvilli, and the cells are joined by junctional complexes. The lateral surfaces of the cells are joined by interdigititating processes, and the basal membrane of the cells shows slight thickening and accumulation of extracellular material similar in appearance to basal lamina (Fig. 4b). In these regions, small membrane-bound vesicles are commonly found (Fig. 4b).

Type 3 Cells
Phase Contrast. These are plump spindle-shaped cells which grow as aligned sheaves or swathes between areas of other cells (Fig. 5, inset).

Electron Microscopy. These are elongated cells which occur in groups, with their long axes running parallel with each other (Fig. 5). Where they are exposed to the culture medium, they have small microvilli on their surface membrane. Lateral membranes of adjacent cells have interdigititating processes and well-developed desmosomes. Cytoplasmic organelles and nuclei resemble those seen in type 1 cells. However, bundles of cytoplasmic filaments are sometimes present immediately underlying the cell membrane, running along the long axis of the cell, and may correspond to the actin filaments previously described (10).

Type 4 Cells
Phase Contrast. These cells have been seen to develop from type 3 cells by time-lapse photography (10). They are distinguished from type 3 cells by being more elongated and by having multiple vesicles at the cell membrane.

Electron Microscopy. Cells corresponding to this phase-contrast description were not recognized in the present study. However, the presence of intercellular spaces in some cultures rather than intracellular vacuoles may explain the phase-contrast observations of this cell type.

Type 5 Cells
Phase Contrast. These cells are larger and less refractile than type 2 cells and are characterized by having numerous small vesicles within their cytoplasm (Fig. 6).

Electron Microscopy. Most easily recognized in the trypsinized pellet, these cells are characterized by large numbers of vacuoles in the cytoplasm (Fig. 6). The surface membrane has few microvilli, and the cell has no consistent polarization except the eccentric positioning of the nucleus, which is irregular with prominent nucleoli. The mitochondria are more electron dense in these cells and may exhibit dilated cristae. The vacuoles have contents varying from very little to large numbers of small vesicles (Fig. 6, inset). While some of the vesicles appear to be derived from enlarged autolysosomes, this is clearly not so in many of the cells. The cells may contain considerable areas of slightly dilated smooth endoplasmic reticulum.

Type 6 Cells
**Phase Contrast.** These cells occur scattered throughout the monolayer and contain large, fluid-filled vacuoles. These cells were more commonly observed in floating cords of cells than in monolayers (Figs. 7 and 11).

**Electron Microscopy.** Usually clustered together, these cells contain one large vacuole (Fig. 8). The exposed cell membranes have surface microvilli, and the cytoplasm has few organelles with a rounded nucleus.

**Type 7 Cells**

**Phase Contrast.** These syncytial cells are found scattered throughout the monolayer and contain from 6 to 20 nuclei (Fig. 9, inset).

**Electron Microscopy.** These are large cells containing numerous irregular nuclei and prominent nucleoli (Fig. 9). The cytoplasm of these cells contains variable quantities of organelles. While some cells contain little but ribosomes and mitochondria, others contain large numbers of electron-dense mitochondria, with dilated cisternae, expanded rough endoplasmic reticulum, and cytoplasmic vacuoles. The surface membrane of the cells forms numerous microvilli.

**Type 8 Cells**

**Phase Contrast.** These cells are found scattered throughout the monolayer and are distinguished by containing lakes of lucent material within their cytoplasm (Fig. 10, inset).

**Electron Microscopy.** Large pale cells are occasionally observed in the cultures with electron-lucent cytoplasm and nuclei lacking peripheral heterochromatin (Fig. 10). These cells appear extremely fragile and often show signs of membrane damage. Their mitochondria have a pale matrix, and the cytoplasm is dominated by large irregular vacuoles with contents varying from, apparently, nothing to large amounts of membranous debris. Unlike the other cell types described, these may be undergoing degenerative and/or senescent changes.

**Free-floating Cords of Cells**

Cells are more rounded than in monolayer, with more rounded nuclei. Ultrastructurally, the cords are characterized by three cell types (Fig. 11).

The first corresponds to the type 2 cells seen in the monolayers and forms lengths of “cuboidal epithelia,” often in two layers “back to back” with their apical membranes exposed to the medium. The basal regions of the cells have small clusters of vesicles, often in short cell processes, with regions of extracellular basal lamina-like material (Fig. 4b). These areas closely resemble breast duct epithelial cells.

The second cell type corresponds to the type 6 cells described above and provides a significant proportion of the cells in the floating cords. These cells are characterized by the presence of a large single vacuole in the cytoplasm (Fig. 11).

The third type of cell seen in the floating cords is not easily recognized in monolayers and may represent a further morphological variant. It is a small cell with a high nuclear:cytoplasmic ratio and very few organelles and is defined by its irregular outline. The cell membrane forms folds, or filopodia, and the tips of neighboring cell processes are joined by desmosomes, giving an appearance reminiscent of basal cells of the skin (Fig. 12).

Microvilli-lined intracytoplasmic lumena were also found in monolayer cultures of PMC42 (Fig. 13).

**Collagen Gel Cultures**

No significant changes in the ultrastructural characteristics of the cell types or degree of pleomorphism were observed in cells grown on collagen gels for periods of up to 10 days, irrespective of whether the gels were relaxed or remained attached to the substrate. The cells in contact with the collagen substratum did not show any novel structures or arrangements.

**DISCUSSION**

A major consideration in the establishment of a cell line developed from a pleural effusion such as PMC42 is the exclusion of mesothelial cells as the cell of origin. The lack of truly specific markers which are capable of unequivocally distinguishing breast epithelial cells from, for example, mesothelial cells makes the identification of PMC42 as of breast epithelial origin dependent upon the accumulation of the results of not one, but a series of investigations.

The reactivity of the milk-fat globule membrane antibodies with “subsets” of cells in the cultures closely resembles that reported for cultures of normal human breast and also breast tumor cell lines (14, 15). Similarly the staining pattern observed with the antikeratin antibody closely resembles that seen in cultures of the breast tumor cell lines (data not shown). This staining pattern is apparent despite the fact that PMC42 appears to lack the large numbers of tonofilaments reported to be present in many
other breast tumor cell lines (12). Furthermore the ultrastructural characteristics of this cell line suggest most strongly that it is derived from breast epithelium, particularly considering the presence of intracytoplasmic microvilli-lined lumina which have been suggested to be a specific characteristic of breast epithelial cell cultures (12).

Similarities in the phase-contrast appearances of PMC42 and normal human breast cultures maintained under similar conditions and the fact that mesothelial cell cultures have been reported not to form multilayered cultures (23) in contrast to PMC42 also indicate the breast epithelial nature of this cell line. Further confirmation comes from the presence of specific estrogen and progesterone receptors and the ability of these compounds to induce a mitogenic response in cultures of PMC42 (24).

The results presented add considerable weight to the previous studies (10, 11, 24), indicating a breast epithelial origin for this cell line, and also confirm the phase-contrast observations that PMC42 exhibits a number of distinctive morphological cell variants in monolayer culture. Of the eight morphological variants described by phase-contrast morphology, at least seven have recognizable ultrastructural counterparts with distinctive features.

The type I cell was previously defined as a "stem" cell for the other cell types in the cultures because of its relatively undifferentiated phase-contrast morphology and its behavior as studied by time-lapse photography (10). Similarly in this study, the majority (but not all) of cell divisions observed took place in areas characterized as type I cells. However, the lineages of the various cell types which can be observed in mature cultures are difficult to deduce from ultrastructural data. Certainly the type I and type II cells appear closely related, as cells with intermediate morphology are readily observed. Elucidation of the interrelationships and possible interconversions between the other cell types requires a more dynamic approach to the problem. We have recently established a series of clones derived from the PMC42 parent line, some of which resemble the parent line, in exhibiting a highly pleomorphic growth pattern, while others show a marked reduction in cellular heterogeneity. These results thus indicate that the parent cell line contains both a population of "multipotential" cells and cells with more restricted differentiative capacities.

These new lines also provide a system with which to identify and analyze the interrelationships which exist between the various cell types and also to undertake strict comparison of the major cell types present in PMC42 and those of both normal human breast and human breast tumors.

Normal human breast epithelium is composed of three basic cell types [epithelial, myoepithelial, and basal clear cells (25-27)], although some minor variations in subdivisions of these main cell types have been reported (28, 29). The different morphological cell types observed within cultures of the breast tumor-derived PMC42 line must therefore be considered within this context.

No obvious counterpart of basal clear cells was observed within cultures of PMC42. It has been suggested that cells answering the description of basal clear cells may form a stem cell population in human breast capable of differentiating into other, functionally mature, cell types (28). However, no ultrastructural similarities were observed between these cells and the type 1 cells in culture of PMC42.

The majority of the cells within cultures of PMC42 do, however, express overt epithelial characteristics. They have microvilli-lined exposed membranes and are joined by junctional complexes at their apical margins, and their lateral membranes often show interdigitating cell processes. Indeed the areas of type 2 cells resemble breast duct epithelium to a striking degree, even to the point of producing basal lamina-like material (particularly in the floating cords of cells) at their basal margins. Such a level of organization has not, to our knowledge, been reported previously with other breast tumor cell lines. The membrane-bound vesicles observed at the base of these cells, in the areas of basal lamina-like material, may be analogous to those described by Stirling and Chandler in the normal breast (27); their functional significance is, however, not known.

The type 3 cells, with their elongated phase-contrast morphology (see Fig. 5), would seem to be the most likely candidates for myoepithelial cells in PMC42 cultures. Ultrastructurally, however, these cells do not present the features of differentiated myoepithelial cells, even when grown on relaxed collagen gels, the technique most likely to induce the expression of both differentiated myoepithelial (30) and secretory epithelial characteristics (31). The failure to observe overt myoepithelial cell differentiation does not, however, preclude the possibility that PMC42 has the capability to form recognizable myoepithelial cells. It may be that the exact in vitro conditions required for the expression and maintenance of this form of differentiation have yet to be defined.

The large vacuoles found in the type 6 cells appear to be derived from a considerable expansion of autophagic vacuoles, as judged by the presence of cell debris inside the vacuoles, but the origins of the vesicles in type 5 cells are not as clear. These vacuoles may be almost empty but more often contain small numbers of regular vesicles (see Fig. 6, inset), and their contents are unknown. Similar structures have been reported in the cell line ZR-75-1, but their contents were interpreted as microvilli with some secretory material (3). Despite the distortion of the cytoplasm of these cells by the presence of large vacuoles, the appearance of the remaining cellular organelles does not support the argument that these cells are terminally degenerated. The processes of lactation and involution are associated with the presence in epithelial cells of large numbers of cytoplasmic membrane-bound vesicles (for review, see Ref 32). Unequivocal biochemical evidence of production of milk proteins and lactose has not been detected in PMC42, rendering the interpretation of the vacuoles as aberrant stages of lactation somewhat speculative, although the finding of electron-dense mitochondria in this cell type is comparable with a previous report (33) of mitochondrial changes during lactation.

Human breast carcinomas exhibit considerable variation in their ultrastructural morphology (34), although recent work (35) has confirmed the expression of an epithelial rather than myoepithelial phenotype. The presence of several distinctive morphological cell types within cultures of PMC42 makes this cell line unique among human breast carcinoma cell lines as a system capable of exposing, for detailed analysis, structural and functional differentiation within human breast carcinomas.

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Fig. 1. Immunofluorescent localization of membrane and cytoplasmic antigens in confluent cultures of PMC42 using LICR LON M3 (a, × 1,200); LICR LON M24 (b, × 1,200); LICR LON M18 (c, × 1,200); and antikeratin antibodies (d, × 2,500).
Fig. 2. a, PMC42 monolayer culture showing cellular pleomorphism. Phase contrast, x 150. b, normal human breast epithelium grown under the same conditions as PMC42 (see Fig. 2a). Phase contrast, x 150.

Fig. 3. Electron micrograph. Type 1 cells from a monolayer, showing relatively small undifferentiated cells. Most cell divisions were observed in areas of this cell type. x 4,400. Inset, phase-contrast micrograph of monolayer culture of PMC42 showing undifferentiated type 1 cells. x 170.
Fig. 4. a, electron micrograph. Type 2 cells from a trypsinized pellet. These well-differentiated polarized cells form structures resembling ductal epithelium. Microvilli are present on the exposed upper membranes, and basal lamina-like material (arrow) is present at the basal regions of the cells. The cell marked with a star is showing signs of degeneration. × 6,000. b, electron micrograph. Type 2 cells in monolayer and in floating cords of cells produce extracellular basal lamina-like material. Often the cytoplasm of the cells adjacent to extracellular basal lamina contain numerous small membrane-bound vesicles. × 30,000. c, phase-contrast micrograph of monolayer culture of PMC42 showing cuboidal type 2 cells. × 170.

Fig. 5. Electron micrograph. Type 3 cells from a monolayer; elongated cells with some surface microvilli. × 4,600. Inset, phase-contrast micrograph of monolayer culture of PMC42 showing elongated type 3 cells. × 170.
Fig. 6. Electron micrograph. Type 5 cells from a trypsinized pellet; large numbers of cytoplasmic vacuoles characterize this cell type. × 4,000. Inset, electron micrograph. The contents of the vacuoles in some type 5 cells consist of large numbers of small vesicles. × 18,000.

Fig. 7. Phase-contrast micrograph of monolayer culture of PMC42. Type 5 cells contain many small vacuoles (short arrow), and type 6 cells contain one large vacuole (long arrow). × 200.

Fig. 8. Electron micrograph. Type 6 cells from a floating cord of cells. Most numerous in the floating cords of cells, these cells contain one large vacuole, usually containing small amounts of membranous debris. × 3,800.

Fig. 9. Electron micrograph. Type 7 cells from a trypsinized pellet, consisting of large multinucleate cells with numerous cytoplasmic organelles. × 2,900. Inset, phase-contrast micrograph of monolayer culture of PMC42 showing large multinucleate type 7 cells (arrow). × 140.
Fig. 10. Electron micrograph. Type 8 cells seen here in a floating cord of cells; they are large, pale, and fragile cells with large irregular vacuoles in the cytoplasm. × 2,500. Inset, phase-contrast micrograph of monolayer culture of PMC 42 showing type 8 cells with large cytoplasmic "lakes" (arrow). × 240.

Fig. 11. Electron micrograph. Low-power view of a floating cord of cells showing the different cell types. × 1,000.

Fig. 12. Electron micrograph. Cells found in the center of floating cords are separated by large intercellular spaces and are joined by desmosomes on adjacent cell processes. × 10,500.
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