Ultrastructural Differentiation of a Clonal Human Embryonal Carcinoma Cell Line in Vitro

Ivan Damjanov and Peter W. Andrews

ABSTRACT

A cloned human embryonal carcinoma (EC) cell line 2102Ep derived from a testicular teratocarcinoma was characterized by means of electron microscopy and immunohistochemistry. These EC cells when plated at high cell density grow mostly as undifferentiated cells displaying relatively little pleomorphism. Eighty-five to 90% of these cells contain keratin in the form of peridesmosomal tonofilaments. Cell populations of the same clonal line plated at a low cell density contain, in addition to undifferentiated EC cells, large cells displaying complex cytoplasmic architecture, more complex junctions, and intracytoplasmic keratin in the form of bundles. Some of these cells also react with antibodies to human chorionic gonadotropin indicative of trophoblastic differentiation. Furthermore, some cells form "morules" which are multicellular aggregates composed of a core of EC cells and an attenuated, more differentiated outer cell layer. These data thus point out not only some similarities but also even more prominent differences between human and mouse EC cells.

INTRODUCTION

Teratocarcinomas are malignant tumors composed of rapidly proliferating undifferentiated stem cells and various tissues originating from them (31).

EC cells, the stem cells of teratocarcinomas, have been studied extensively in the mouse tumor model (1, 12, 15, 20, 22, 33), but human EC cells have, until recently, been unavailable for more complex studies requiring an isolated, homogeneous, and defined cell population. Recently, several cell lines derived from human teratocarcinomas have been established as xenografts in immunodeficient animals (17, 24, 30, 35) or adapted to in vitro growth (8, 11, 13, 18, 36-38). Data from these in vitro-established human cell lines have suggested that these human tumor cells have many similarities with the mouse teratocarcinoma cells but are also different in many respects (2-7). This has led us to propose that the human EC cells might correspond to a developmentally different population of undifferentiated embryonic cells than would the mouse EC cells and that the data obtained in the mouse tumor cell system should not be transposed directly to the human condition (7, 14). However, since none of the presently available human cell lines was cloned, doubts still persist as to whether the differences between the mouse and human cells were inherent to cells of the 2 species or due to the heterogeneity of cell populations isolated from human tumors.

Andrews et al. (6) have cloned recently and characterized an EC cell line established in vitro from a human testicular teratocarcinoma. This cell line, 2102Ep, if grown in cultures seeded at high cell density, consists of a uniform population of undifferentiated cells which, upon injection into an immunodeficient animal, form tumors morphologically indistinguishable from typical human ECs. When these same cells are plated in vitro at a low cell density, they give rise to mixed populations consisting of morphologically distinct large cells as well as of small, undifferentiated EC cells that form the bulk of the cell population in cultures plated at high cell density. The large cells differ from the parental stem cells not only morphologically but also with regard to cell surface markers. They also synthesize fibronectin, whereas the undifferentiated cells do not (2). Morphological and antigenic changes observed in cell culture have been interpreted as signs of limited differentiation (6, 9, 26, 32). In this paper, we provide ultrastructural evidence that the large cells indeed represent distinct derivatives of the typical undifferentiated EC cells. Furthermore, we show that the cells of this cloned human EC line form complex intercellular junctions and multicellular structures and most probably represent some other forms of differentiation in culture. All these findings are juxtaposed and compared with the findings in mouse EC cells grown in vitro.

MATERIALS AND METHODS

The human cell line 2102Ep was derived from a surgical specimen of a primary testicular teratocarcinoma containing EC cells and elements of yolk sac carcinoma and various somatic tissues (4, 36). Clonal cultures 2A6 and 4D3 were isolated by picking single cells with a micropipet viewed under a dissecting microscope (6). All the cells were grown in tissue culture plastic flasks (Falcon) at 37°C in Dulbecco's modified minimal essential medium (high glucose formulation; Flow Laboratories, Rockville, Md.) supplemented with 10% heat-inactivated fetal calf serum (Reheis, Armour Pharmaceuticals, Chicago, Ill.) in a humidified atmosphere containing 5% CO₂ in air. They were harvested and subcultured by brief treatment with 0.25% trypsin and 2 mM EDTA in Dulbecco's phosphate-buffered saline lacking calcium and magnesium. High-density stock cultures of the cloned cells were maintained by plating 5 × 10⁶ cells into 75-cm² flasks and subculturing every 3 to 4 days. Low-density cultures were prepared by plating cells from a high-density stock culture at 10⁶ cells/75-cm² flask. Cells from high-density cultures were harvested for further studies 3 to 4 days after the initial plating, and the cells from low-density cultures were harvested 5 to 7 days after the seeding.

Electron Microscopy. Cells were harvested following a brief treatment with 0.25% trypsin and were fixed in 2% buffered glutaraldehyde, postfixed in 1% buffered osmium tetroxide, dehydrated in alcohol, and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and studied with a Zeiss Model EM9 electron microscope.

Immunohistochemistry. Monolayer cultures grown in multichamber
Lab-Tek tissue culture slides were fixed with cold acetone and reacted with rabbit anti-keratin and anti-vimentin antibodies diluted 1:50 followed by the secondary FITC-labeled goat anti-rabbit immunoglobulin antibody diluted 1:20 (Cappel Laboratories, Cochranville, Pa.). The antikeratin and antivimentin sera were kindly supplied by Dr. T. T. Sun and Dr. E. Frank. The specificity of these antibodies has been tested and described previously (16, 34). Appropriate controls including absorption with human skin-extracted keratin and fibroblast-extracted vimentin were performed as described previously (16, 34).

Cells grown at high or low density were harvested in the usual way, pelleted by brief centrifugation, and fixed for 24 hr in Bouin's fixative followed by postfixation in 70% ethanol. These pellets were then dehydrated and embedded in paraffin wax and sectioned with a microtome. For staining, sections were cut and deparaffinized in xylene and alcohol, rehydrated, and washed extensively in 0.9% NaCl solution. They were then reacted with FITC-labeled rabbit anti-human α-fetoprotein (DACO) diluted 1:10. Other sections were reacted with rabbit anti-HCG, β-chain specific and diluted 1:1000, provided by F. T. Bosman (10), followed by FITC-labeled goat anti-rabbit immunoglobulin diluted 1:20.

RESULTS

Electron Microscopy of High-Cell-Density Cultures. Most of the cells harvested from flasks plated at high density were of relatively uniform size, measuring an average of 12 to 15 μm in diameter. These cells displayed a high nucleocytoplasmic ratio with the nucleus being relatively large and located centrally and the cytoplasm forming a perinuclear rim of uneven width (Fig. 1). The nuclei were mostly shaped irregularly, indented deeply, or even multilobulated due to the deep indentations of the nuclear membrane. Nucleoli were large and often multiple. Euchromatin predominated over heterochromatin. The cytoplasm contained few organelles which usually included a few mitochondria, occasional profiles of rough endoplasmic reticulum, and prominent free ribosomes. Slender microvilli extended from the surface of the plasma membrane and protruded into the frequently dilated intercellular processes. The juxtaposed sides of closely adjacent cells were interconnected with one another by intercellular junctions corresponding to terminal bars, gap junctions, and poorly developed desmosomes. No extracellular matrix was seen. In addition to these highly undifferentiated neoplastic cells, there were at the periphery of cell groups a few cells showing more cytoplasm and thus with a lower nucleocytoplasmic ratio than that of the predominant cell type in culture (Fig. 2). The cytoplasm of these cells contained few organelles, but the intercellular junctions on the cell surface were more prominent and better developed. They often appeared to be polarized because of the eccentric location of their nucleus and the aggregation of the microvilli on one side of the plasma membrane (Fig. 2). Although devoid of cytoplasmic organelles in general, the cells characterized by more abundant cytoplasm frequently contained bundles of intermediate filaments in their cytoplasm as well as attached to the desmosomes (Fig. 2). These cells also had less prominent nucleoli than did the undifferentiated EC cells, and frequently no nucleoli were seen in a particular section (Fig. 2). Larger cells with more cytoplasmic organelles resembling those found in the cell cultures plated at low density were seen only rarely.

Electron Microscopy of Low-Cell-Density Cultures. These cultures contained the same cells as seen in flasks seeded at high cell density but also numerous large cells measuring up to 50 μm in diameter. Aggregates of tumor cells often showed a gradient, the small cells being on one side and the large cells being on the other side of cells that were of intermediate size (Fig. 3). Tight junctions and desmosomes were well developed, and the intracytoplasmic filaments were more abundant (Figs. 4 and 5). Many of the large cells contained not only more cytoplasm than did the typical EC cells but also many more organelles, most notably mitochondria, membrane-bound lysosomal granules, and large osmiophilic membrane-bound granules (Fig. 6). Giant cells with the cytoplasm filled with glycogen, keratin filaments or smooth endoplasmic reticulum, and mitochondria with vesicular cristae were considered to represent syncytiotrophoblastic cells (Fig. 7). No intercellular matrix was seen. Many of the large cells showed degenerative changes such as myelin figures, residual bodies, and extensive vacuolization. There were also many disintegrated and completely necrotic cells in the culture.

The cell suspension submitted for electron microscopy also contained aggregates of 3 to 8 cells composed of a central core of typical undifferentiated EC cells surrounded on one side by elongated or flattened cells of a somewhat different morphology (Fig. 8). The flattened cells had a partially vacuolated cytoplasm filled with mitochondria, free ribosomes, and short profiles of rough endoplasmic reticulum. The 2 cell types were separated from one another by an intercellular space and were only rarely peripherally interconnected with desmosomes or tight junctions. The external plasma membrane of the elongated cells formed prominent microvilli.

Immunohistochemistry. Immunohistochemical reaction with the antibodies to keratin disclosed that 80 to 85% of all cells in the high-cell-density cultures contained immunoreactive keratin (Fig. 9, a and b). In most cells, the antibody reacted with the filaments along the plasma membrane, although in addition, some indistinct cytoplasmic staining could be seen in most cells. The cultures also contained groups of cells with prominent meshworks of cytoplasmic filaments (Fig. 9b). These filaments reacted only weakly with the anti-keratin antibody but also with the nonimmune rabbit serum and thus most probably represented the “tension filaments” of actin (21, 28).

Immunohistochemical reaction of cells plated at low cell density disclosed immunoreactive keratin in 90 to 95% of cells (Fig. 9c). Most cells still showed reactivity along the plasma membrane. However, there were also numerous cells with a delicate cytoplasmic meshwork that reacted with antibodies to keratin. At the periphery of cell nests, there were also numerous highly keratinized cells (Fig. 9c) which were only exceptionally seen in some of the high-cell-density cultures.

Immunohistochemical reaction with the antibody to vimentin labeled 15 to 20% of cells in cultures plated at high cell density and 30 to 35% of cells in cultures plated at low cell density (Fig. 9d). Most of the immunoreactive cells stained intensely and disclosed immunoreactive keratin in 90 to 95% of cells (Fig. 9d). Many of the large cells contained not only more cytoplasm than did the typical EC cells but also many more organelles, most notably mitochondria, membrane-bound lysosomal granules, and large osmiophilic membrane-bound granules (Fig. 6). Giant cells with the cytoplasm filled with glycogen, keratin filaments or smooth endoplasmic reticulum, and mitochondria with vesicular cristae were considered to represent syncytiotrophoblastic cells (Fig. 7). No intercellular matrix was seen. Many of the large cells showed degenerative changes such as myelin figures, residual bodies, and extensive vacuolization. There were also many disintegrated and completely necrotic cells in the culture.

In a previous report, we have described the cloning of the human EC cell line 2102Ep (6). The strict criteria for cloning...
applied in that work, coupled with the successful propagation of
the cells for more than 50 transfer generations without any
considerable change in their morphology, indicate that we are
indeed dealing with a stable clonal line. The facts that the tumor
was isolated from a human testicular teratocarcinoma, that
the cells injected into nude mice produce solid tumors indistinguish-
able from EC, and that the cells express SSEA-3 which is the
typical marker of human EC cells (14) all indicate that cell line is
indeed an EC. The present ultrastructural findings further con-
firm this contention, showing that the in vitro-established line does
not differ from EC cells described previously found in solid tumors
or from other clonal lines of EC established as xenografts (24). A
human EC cell line isolated from an ovarian teratocarcinoma
showed many ultrastructural similarities with the present tumor
cell line (38), indicating that the EC cells, irrespective of their
origin, retain the same morphology.

The present data give additional evidence of limited differen-
tiation of this clonal EC line. In concordance with the findings of
Pierce (29) who has described considerable ultrastructural mor-
phological variation in the appearance of EC cells in solid EC
tumors in vivo, we have also noticed considerable pleomorphism
of the clonal EC cells, especially in low cell density cultures. The
differences between the EC cell in culture were, however, mostly
quantitative, and although one could clearly distinguish cells with
"empty" cytoplasm from those that contained more organelles,
microvilli, and intercellular junctions, all these cells could be
classified as variants of EC within the range originally recognized
and described by Pierce (29) in solid germ cell tumors.

In addition to these quantitative variations noticed in the EC
cell populations, our findings show unequivocally that the change
of morphology observed by light microscopy indicates a form of
differentiation and that the large and flattened cells are qualita-
tively different from the typical EC cells. In view of the immuno-
reactivity for HCG in some cells, we believe that the differentation
in this cell evolved along the extraembryonic cell lineages (19,
30), i.e., into trophoblastic giant cells. These conclusions, based
on the immunohistochemical findings, are supported by the
ultrastructural data which also suggest that the giant cells with
multilobed nuclei and abundant cytoplasm rich with mitochondria,
rough and smooth endoplasmic reticulum, and lysosomes and
osmiophilic granules are indeed trophoblastic giant cells (29). It
is also possible that some ultrastructurally diagnosed tropho-
blastic cells did not react with the antibody to HCG. Since we
have not performed electron microscopic studies on immunohis-
tochemically reacted cells, we cannot yet resolve this dilemma.

On the other hand, yolk sac carcinoma elements, the other
extraembryonic structure which is frequently seen in mouse EC
cell cultures (22, 23, 33), were not identified positively (19, 25)
in the present study. Neither did we definitively identify the so-
called "embryoid bodies" (23). It should be noticed that the cell
aggregates formed as a core of EC cells, and an attenuated cell
layer resembled mouse embryoid bodies only superficially. These
bodies were therefore called "morules" rather than embryoid
bodies, since we could not establish definitely that the outer
layer was composed of different cells from the core. The outer
layer was not separated from the inner by a basement membrane
which is invariably found in mouse embryoid bodies (23).

Ultrastructural studies on the present tumor material, as well
as those performed on human solid germ cell tumors and xeno-
grafted human ECs (24), indicate that the EC cells are in most
instances interconnected with one another by intercellular junc-
tions. This suggests that the ECs are epithelial cells. The epithe-
lium nature of EC cells is also evidenced by their ability to form
gland-like and papillary structures in solid tumors (29). Our
immunohistochemical data provide additional support to the
contention that the ECs are epithelial cells. Thus, they contain
keratin which is the typical intermediate filament of the epithelial
cells (27, 34). Antibodies specifying keratin have been used suc-
cessfully to differentiate epithelial malignant tumors (carci-
nomas) from mesenchymal tumors (sarcomas) which contain
vimentin instead of keratin as the primary component of their
cytoskeletal filaments (27). The findings of keratin in human EC
cells make these cells quite distinct from mouse EC cells. The
mouse ECs do not contain keratin but rather vimentin as the
cytoskeletal protein of their intermediate filaments (29). This
demonstrates another major biological difference between EC
cells of mice and humans.

Keratin demonstrated in EC cells grown in high cell density
cultures was usually located along the plasma membrane most
probably associated with the intercellular junctions. Intraepi-
plasmic keratin not attached to plasma membrane corresponding
to the filaments seen by electron microscopy was seen only in
cells with more abundant cytoplasm. This contrasts with marked
keratinization of giant cells labeled as trophoblastic. It is known
that mouse trophoblastic cells contain keratin filaments (28). Our
data obtained on Jar and Bewo cells, 2 known trophoblastic
lines, indicate that human trophoblastic cells also contain kerat-
in.4

Vimentin, the cytoskeletal protein or mesenchymal cells (21,
27), was found only in a small number of tumor cells and more
frequently in cells seeded at low density than in those seeded at
high density. Furthermore, vimentin-positive cells were usually
at the periphery of the tumor groups grown for immunohisto-
chemical purposes on histological slides. The nature of these
cells has not been determined, and they could represent either
yolk sac elements, fibroblasts and/or undifferentiated mesen-
chymal cells, or even trophoblastic cells. However, since vimentin
occurs in many epithelial and mesenchymal cells in culture (21),
it is also possible that vimentin occurs in subpopulations of EC
grown in vivo. Future studies will have not only to determine the
nature of vimentin-positive cells in EC cell cultures, but also
whether this intermediate filament is an inherent component of
human EC in vivo or only a consequence of in vitro culture.

Fibroblastic cells have been described previously in cultures
of human EC cells (38), and it is possible that these cells are
indeed fibroblasts. However, since fibroblastic cells occur in
both the somatic tissues and the extraembryonic membranes like
the yolk sac, their finding should not be taken as evidence of somatic
differentiation in the cell culture.

In summary, our ultrastructural and immunohistochemical
studies on a clonal human embryonal carcinoma cell line have
shown that these EC cells can differentiate under appropriate
conditions in vitro. However, the evidence presented here indi-
cates that only extraembryonic cells are formed, and no definitive
sign of somatic differentiation was obtained. In this respect, the
clonal line 2102Ep resembles the mouse cell line F9 which also
can differentiate only to extraembryonic cells (31). The spectrum
of differentiated cells in the human cell line differs from that seen
in the mouse cell line. Thus, although we have shown many
similarities between human and mouse EC cells, we have also

described important differences which appear to be inherent to these cells. Further studies of these differences between human and mouse EC cells are in progress.

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REFERENCES


Fig. 1. High-cell-density culture. The cells have irregularly shaped nuclei, prominent nucleoli, and a high nucleocytoplasmic ratio. × 3,200.

Fig. 2. High-cell-density culture. Cells with more cytoplasm are encountered occasionally. In the cytoplasm, there are mitochondria, a well-developed Golgi apparatus, and a few profiles of endoplasmic reticulum. These cells are interconnected with adjacent cells with tight junctions (arrow) and occasional desmosomes. Cell surface forms villi, usually on one side of the cell membrane (*). Nucleoli are inconspicuous. × 3,400.
Fig. 3. Low-cell-density culture. A group of cells showing considerable variation from one cell to another. × 3,200.

Fig. 4. Low-cell-density culture. Closely adjacent cells interconnected with one another with complex junctions (arrows). Note the paucity of cytoplasmic organelles and the formation of villi on one pole of the cell (†). × 4,800.
Fig. 5. Low-cell-density culture. Cells with abundant cytoplasm. The cytoplasm contains a prominent aggregate of glycogen (G), mitochondria, dense bodies, and bundles of tonofilaments (arrows). × 3,400.

Fig. 6. Low-cell-density culture. Portion of an extremely large cell. Note the irregularly shaped nucleus and abundance of cytoplasmic organelles. × 6,800.
Fig. 7. Low-cell-density culture. Portion of a giant cell. Note mitochondria with vesicular cristae and the abundance of smooth endoplasmic reticulum. × 14,000.

Fig. 8. Low-cell-density culture. Portion of a morule. Note 2 centrally located EC cells (EC) and an attenuated cell surrounding them (arrows). × 3,400.
Fig. 9. Immunohistochemical reaction on high-cell- and low-cell-density cultures. In a to e, photographs were prepared from cells grown on tissue culture slides and mixed in situ, whereas e' was prepared from cell suspensions embedded in paraffin. a, high-density culture reacted with antibody to keratin. Note the reaction along the plasma membrane, most probably corresponding to intercellular junctions. × 320. b, high-density culture reacting with antibodies to keratin. Groups of cells show reaction products not exclusively along the intercellular junctions and outlines of tension filaments in the cytoplasm. × 320. c, low-cell-density culture reacting with antibodies to keratin. All cells contain immunoreactive filaments. Cells at the periphery are keratinized densely. × 220 (underexposed because of the heavy reactivity of cells at the periphery). d, low-cell-density culture reacting with antibodies to vimentin. Approximately 40% of the cells reacted with the antibody. × 160. e and e', low-cell-density culture reacting with the antibody to HCG. Only a few cells show cytoplasmic reaction, whereas all the others are nonreactive. e, × 220; and e', × 380.
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