Scanning Electron Microscopic Observation of Two Retinoblastoma Cell Lines

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ABSTRACT

Two continuous retinoblastoma cell lines were observed by scanning electron microscopy. Both cell lines spontaneously grow as a suspension of round cells in clusters, chains, and unique ring (rosette) formations. Scanning electron microscopy of suspension cells reveals some variation in the number and frequency of surface adornments such as blebs, lamellipodia, and microvilli. Although the cell lines are nonadherent to substratum and therefore assume a spherical form, highly villous cells are not characteristic of the entire cell populations.

When WERI-Rb1 and Y79 are seeded onto a polyornithine-treated substrate, attachment and growth as adherent cultures are evident. With selective attachment on a positively charged substrate, we observe alteration of membrane architecture with the extension of cytoplasm and filopodia. In addition, WERI-Rb1 cell-to-substratum adhesion induces morphological changes suggestive of neuronal cell differentiation.

INTRODUCTION

Retinoblastoma is the most common intraocular cancer of childhood, occurring with a frequency of about 1 in 20,000 births. Small, round cells with scanty cytoplasm and large hyperchromatic nuclei comprise undifferentiated tumors. The rosettes or fleurettes observed in differentiated tumors reveal the histogenesis of retinoblastoma. Tso et al. (18) have shown by transmission electron microscopy that the cells constituting rosettes contain cell processes resembling photoreceptor elements. More recently, Ohnishi (9) conducted an extensive investigation of the rosettes within clusters, chains, and unique ring (rosette) formations. In contrast, Y79 cells grow in grape-like clusters, chains, and unique ring formations. In contrast, Y79 cells grow in grape-like clusters, chains, and unique ring formations.

Unlike other tumor-derived cell lines, neither WERI-Rb1 nor Y79 exhibits adhesiveness or growth as a monolayer on negatively charged plastic tissue culture flasks. The inability to form monolayers, i.e., loss of cell-to-substrate adhesiveness, may be related to the quantity and distribution of negatively charged cell membrane components. The attachment and growth of WERI-Rb1 and Y79 on positively charged polyornithine-treated flasks, as previously described by McFall et al. (7), suggests that retinoblastoma cells have uniquely charged membranes.

In order to determine the extent of alteration in cell morphology during attachment and growth of WERI-Rb1 and Y79 on a modified substrate, we observed suspension and adherent cultures of the retinoblastoma cell lines with SEM.

MATERIALS AND METHODS

Suspension Cultures. Replicate 30-ml plastic tissue culture flasks (Falcon Plastics, Oxnard, Calif.) were seeded with 5 × 10⁵ WERI-Rb1 or Y79 cells in 5 ml of Roswell Park Memorial Institute Medium 1640 supplemented with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.). The flasks were set upright for suspension growth and incubated at 37°C in a 95% air-5% CO₂ humidified atmosphere. Medium was replaced once during the test period.

Adherent Cultures. Growth of adherent cells was accomplished with a modification of the bovine serum albumin polymer technique of Macieira-Coelho and Avrameas (6). Replicate 30-ml plastic tissue culture flasks were treated with a solution of polyornithine as previously described by McFall et al. (7). The flasks were then seeded with 5 × 10⁶ WERI-Rb1 or Y79 cells in 5 ml of nutrient medium, placed in the normal horizontal position for monolayer growth, and incubated. Medium was replaced once during the test period.

Preparation of Cells for SEM. Suspension cultures of WERI-Rb1 and Y79 at 1, 3, 5, 7, 9 and 11 days postseeding were pelleted and washed in warm (37°C) phosphate-buffered saline (NaCl, 8 g/liter; KCl, 0.2 g/liter; Na₂HPO₄, 1.15 g/liter; KH₂PO₄, 0.2 g/liter; CaCl₂, 0.1 g/liter; and MgCl₂, 0.1 g/liter). The final pellet was resuspended in 1 ml of warm (37°C) 2.5% glutaraldehyde buffered to pH 7.2 with 0.1 M sodium cacodylate-HCl, drawn into a syringe, and gently passed through a Swinex adapter containing a 13-mm x 1.2-µm silver membrane filter (Selas Flotronics, Spring House, Pa.). A small amount of fixative was allowed to remain in the adapter for 5 min, after which the filter (to which cells were...
adhering) was removed and placed in a beaker of fresh fixative for an additional 30 min. After fixation the cells were washed in 0.2 M sodium cacodylate-HCl buffer for two 5-min intervals. Specimen dehydration was accomplished at 5-min intervals in graded ethanol (30, 50, 70, 90, 100, and 100%), ethanol-amyl acetate (50 to 50%), and finally amyl acetate (100%). While submerged in amyl acetate, the cells were processed through a Sorvall Critical Point Drying Apparatus (Ivan Sorvall, Inc., Newtown, Conn.) with the use of CO2 according to the technique described by Anderson (1). The filters were then placed on aluminum studs and coated with carbon and gold in an Edwards 306 vacuum evaporator. The cells were examined with a Joel JSM 50A scanning electron microscope at 25 kV with the specimen tilted at 0°, 30°, or 45°.

Adherent cultures of WERI-Rb1 and Y79 at 1, 3, 5, 7, 9, and 11 days postseeding were washed with phosphate-buffered saline and fixed for 30 min in warm (37°C) 2.5% glutaraldehyde buffered to pH 7.2 with 0.1 M cacodylate-HCl. After primary fixation the cells were washed twice in cacodylate-HCl buffer, postfixed for 30 min in 0.5% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, Pa.) in 0.2 M cacodylate-HCl buffer, and then washed with 0.2 M cacodylate-HCl buffer. While submerged in buffer a 20- x 10-mm section was cut (with a "hot" pen) from the growth surface of each flask. Dehydration of the adherent cells was accomplished at 5-min intervals in graded ethanol (30, 50, 70, 90, 100, and 100%). While submerged in 100% ethanol, the specimens were processed through a Sorvall Critical Point Drying Apparatus, coated with carbon and gold, and examined as previously described.

RESULTS

SEM Suspension Cells. WERI-Rb1 and Y79 suspension cells are pseudospherical to spherical and are approximately 7 μm in diameter. Cells are arranged in clusters, in unique ring (rosette) formations, or in chain formations, (Fig. 1) or they appear singly (Fig. 2). Some variation in cell size and in the number and frequency of surface adornments such as blebs, microvilli, and lamellipodia is evident. The most frequently observed surface excrescences are blebs. These bulbous or spherical “exocytotes” range in size from 0.1 to 1 μm in diameter. Some blebs rest on the cell surface, while others are attached to the cell by a pedicle. Blebs are most numerous on WERI-Rb1 surfaces immediately after initiation of cultures and again at 3, 7, and 11 days postseeding. Y79 cells exhibit increased blebbing immediately after seeding and again at 1, 3, and 5 days. Interestingly, many retinoblastoma cells have surface activities suggestive of normal cultured cells; their membranes are extraordinarily free of surface excrescences (Fig. 3).

Although WERI-Rb1 and Y79 spontaneously grow in suspension and therefore assume a rounded morphology, highly villous cells are not characteristic of the entire cell population. While highly villous cells are evident, they comprise only 20% of all cell populations examined. Generally, cells show an occasional microvillus, clusters of microvilli, or none at all. Occasionally, cells are covered with short finger-like protrusions, while other cells have longer surface extensions. Microvilli are approximately 0.1 μm in diameter and range in length from 1 to 6 μm. The long surface excrescences appear tortuous and marked by repeated twists, bends, and localized swellings along their length. This type of morphology is characteristic of the microvilli of highly villous cells (Fig. 4a).

SEM of Adherent Cells. Immediately after cell seeding, WERI-Rb1 and Y79 cells adhere to polystyrene-treated flasks. Generally, adherent cells remain spherical, and they appear singly, in clusters, and in chains and rosettes. Surface excrescences such as blebs and microvilli seem to occur with the same frequency as do those observed on suspension cells. However, lamellipodia in the form of ruffles appear to be characteristic of adherent cells. Like suspension cells, some adherent cells are relatively unadorned. Surface alterations in the form of filopodia are evident with the attachment of cells to a positively charged substrate. The slender tentacular processes emanate from the cell margins in contact with the substrate; they are observed on all adherent cells and probably serve to anchor the cell to the substrate. Filopodia vary in length and diameter. In addition some have bulbous expansions along their length or at their tips. Although filopodia usually appear singly, the arborization of the long processes extending from the lateral margins of some cells is evident. The extensive branching of the distal ends of filopodia is typical of WERI-Rb1 cells (Fig. 7) while the filopodia associated with Y79 appear shorter and less complex. In some instances, spike-like adornments are observed extending from Y79 cells (Fig. 15).

The attachment of some WERI-Rb1 and Y79 cells is accompanied by the formation and spread of flattened areas of cytoplasm upon the substrate (Figs. 12 and 15). In most instances filopodia emanate from the leading edges of flattened cytoplasm (Fig. 15). Although the spread of cytoplasm is evident, this area remains sparse and the central cell body maintains a spherical form. The greatest degree of cytoplasm spreading is observed with the 9- and 11-day cultures of WERI-Rb1. Some cells show loss of spherical form with cellular elongation and concomitant morphological alterations in the form of axon-like processes (Fig. 10) which terminate in completely flattened structures reminiscent of neuronal cell bodies with dendrites (Fig. 11).

The expression of cell growth in chains and rosettes is an interesting phenotypic characteristic of WERI-Rb1 and Y79. Adherent culturing permits better visualization of retinoblastoma cells in these unique formations. Initially, after cell seeding on the modified substrate, WERI-Rb1 cells appear singly, in clusters, or in short chains; however, by Day 11, cells are observed in a complex network of intertwining chains and rosettes (Figs. 5 and 6). In contrast Y79 chain formations are less dramatic, usually consisting of only 4 to 5 cells (Fig. 13). Generally, WERI-Rb1 rosettes consist of 8 cells in ring formation around a central lumen (Figs. 8 and 9). In contrast, the rosettes characteristic of Y79 cultures usually consist of 5 cells in a unique ring formation (Fig. 14). Variation in the surface activities of the cells comprising rosettes is observed. Some cells are adorned with a few blebs and microvilli (Figs. 8 and 14), while the cells comprising other rosettes have highly ruffled membranes (Fig. 9). Cells in rosette formations apparently function as a unit;
all cells have identical surface activities at a given time. Anchorage of rosettes to substrate is usually accomplished by means of short filopodia (Fig. 8).

In dense adherent cultures of Y79 (7 and 9 days postseeding), the cells pile up on one another. At this time the cells become totally spherical and lose their tendency to spread cytoplasm. Y79 cells in contact with substrate as well as those comprising cell clumps display short filopodia. When adherent cultures of WERI-Rb1 and Y79 (11 and 9 days, respectively) reach saturation density, some cell detachment from the substrate occurs.

DISCUSSION

The degree to which cells in culture adhere to substrate is an important cellular property. Attachment of normal cells to a suitable substrate is usually necessary to initiate cell proliferation. With SEM it has been shown that normal cell-to-substratum adhesion is accompanied by the flattening and spreading of cytoplasm (2, 3, 14). Although most neoplastic cells in culture attach and grow as monolayers, they exhibit decreased adhesiveness and flattening of cytoplasm upon a negatively charged substrate. It has been suggested that decreased neoplastic cell-to-substratum adhesion results from deficient or incomplete lamelliplasm formation (3). Changes in the surface properties of neoplastic cells, such as loss of high-molecular-weight glycoprotein after transformation, probably play an important role in the behavior of cells in vitro (20, 21).

WERI-Rb1 and Y79 are characterized by a rounded morphology and growth in suspension. Unlike other tumor-derived cell lines, neither WERI-Rb1 nor Y79 grows as monolayer on plastic tissue culture flasks. These flasks carry a negative charge. The selective attachment of these cells lines on a positively charged (polyornithine-treated) substrate suggests that the mechanisms of retinoblastoma cell adhesion differs from that of other malignant cells in culture. The quantity and distribution of negatively charged membrane components on retinoblastoma cell lines may differ significantly from those of other neoplastic cells.

With attachment we observe growth control and some alteration of cell surface architecture when WERI-Rb1 and Y79 are cultured on a modified substrate. Adherent cells appear as spheres attached to substrate by filopodia or as spheres slightly flattened by the spreading of cytoplasm. Furthermore, WERI-Rb1 cell-to-substratum adhesion induces morphological changes suggestive of neuronal cell differentiation. With attachment on a modified substrate, retinoblastoma cells express a decreased growth rate (7). Since WERI-Rb1 characteristically exhibits decreased growth in suspension (as compared to the more anaplastic Y79), attachment on a positively charged substrate could restrict the rate of cell division significantly and thus permit uninterrupted synthesis of axons and cell bodies. Neurite extension by neuroblastoma cell lines is greatest when serum depletion from the culture imposes restrictions on cell division (17).

The types of surface adornments that we observe on WERI-Rb1 and Y79 suspension cells (blebs, lamellipodia, microvilli) have been described on other cells in culture (10–13). However, SEM reveals that retinoblastoma cells have certain surface characteristics that distinguish them from other neoplastic cells in vitro. Variation in the number and frequency of blebs and lamellipodia may be characteristic of certain phases in the division cycle or the response of the retinoblastoma cell to its environment (4, 12). Observation of cells that are totally free of surface adornments is surprising, since it has been suggested by Porter and Fonte (10) that most cancer cells probably display some surface excrescences throughout the cell cycle. In contrast, Porter et al. (12) have shown that normal proliferating cells in late G, and S are free of surface adornments. Our observations of retinoblastoma cells that are free of surface adornments support another suggestion by Porter and Fonte (10) that the surface activities of some tumorigenic cells may resemble those of normal cells.

The association of microvilli with the malignant transformation of cells is well documented (5, 11, 16, 20). In addition, it has been concluded that, as a result of decreased cell-to-substrate adhesiveness, malignant cells assume a rounded morphology, microfilament bundles disappear, and microvilli and ruffles become more numerous (19). It is surprising that only a small percentage of WERI-Rbl and Y79 suspension cells are highly villous. Our observations of microvilli-free as well as highly villous, nonadherent retinoblastoma cells suggest that there is no correlation between a rounded morphology, microvilli, and nonadhesiveness within these cell lines. In addition, our observation suggests that microvilli are not always associated with malignant cells in culture (5).

SEM of suspension and adherent cell cultures affords an opportunity to observe the morphology of WERI-Rb1 and Y79 cells in unique chain and rosette formations. While these rosettes are not presumed to be identical with the type described in differentiated retinoblastomas, it is speculated that they are an expression of in vitro differentiation. We also speculate that chain formations are an intermediate form of rosettes. Ohnishi (8) has demonstrated that short-term cultures of dissociated embryonic retinal cells can reaggregate to form rosettes. Since retinoblastoma arises from an embryonic retinal cell, it may be possible that rosette formation by cell lines derived from these tumors is an expression of in vitro differentiation.

WERI-Rb1 and Y79 cell lines exhibit several unique in vitro characteristics: spontaneous growth in suspension; selective attachment on a positively charged substrate; adherent cell-to-substratum differentiation; and growth in rosette and chain formations. Further investigation will be necessary to determine the mechanisms responsible for the in vitro behavior of retinoblastoma cells. Association of a biochemical function with the morphological differentiation observed in suspension and adherent cultures is presently being investigated.

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REFERENCES

1. Anderson, T. F. Techniques for the Preservation of Three-Dimensional


Fig. 1. WERI-Rb1 suspension cells in chain formation. Day 5 postseeding.
Fig. 2. The membrane of this Y79 suspension cell is adorned with ruffles (L) and blebs (B). Day 1 postseeding.
Fig. 3. The membranes of these Y79 suspension cells are extraordinarily free of surface adornments although a few blebs (B) are evident. Day 5 postseeding.
Fig. 4. Y79 suspension cells. a, a highly villous cell; b, a cell with no surface excrescences. Day 7 postseeding.
Fig. 5. WERI-Rb1 cells in a complex network of chains and rosettes (arrows) are illustrated in this micrograph of an adherent culture. Day 11 postseeding.

Fig. 6. The intertwining of cell chains is apparent in this higher magnification of Fig. 5.

Fig. 7. This cluster of 5 WERI-Rb1 adherent cells is anchored to the substrate by numerous, arborizing filopodia (arrows). Day 7 postseeding.

Fig. 8. WERI-Rb1 cells are observed in a unique rosette formation. The cell membranes are adorned with blebs, microvilli, and filopodia. Day 7 postseeding.
Fig. 9. In contrast to Fig. 8, the cells comprising this WERI-Rbl rosette have highly ruffled (R) membranes. Day 9 postseeding.

Figs. 10 and 11. These micrographs of an WERI-Rbl cell dramatically illustrate differentiation during adherent cell culturing. The cell shown in Fig. 10 branches into 2 axon-like structures which terminate in a flattened process reminiscent of a neuronal cell body with dendrites (Fig. 11). Day 11 postseeding.
Fig. 12. Cytoplasm (La) spreading during adherent culturing of Y79 cells. The membranes of these 3 cells show a few surface excrescences. One microvillus (M) gives a "vein-like" appearance. Day 1 postseeding.

Fig. 13. This short chain of cells is typical of those observed in Y79 cultures. Filopodia anchor these cells to substrate. Cytoplasm in the form of a pseudopodium (arrow) appears to have broken off 1 cell. Day 3 postseeding.
Fig. 14. The cells comprising this unique Y79 rosette are adorned with a few microvilli while filopodia anchor the rosette to the substrate and attach from cell to neighboring cell. Day 3 postseeding.

Fig. 15. Attachment of these Y79 adherent cells to substrate is affected through cytoplasm (La) spreading, spike-like filopodia (sF), and filopodia (F) which emanate from the cytoplasm. Day 5 postseeding.
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