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Retinoblastoma Y79 Cell Line: A Study of Membrane Structures

Andrea L.W. Green[1], Edward S. Meek[1,3], David W. White[2], Reggie H. Stevens[2], Larry D. Ackerman[5], George F. Judisch[1], and Shivanand R. Patil[4]

Departments of Ophthalmology[1], Radiology (Radiation Research Laboratory)[2], Pathology[3], Pediatrics[4], University of Iowa; and Research and Development Service[5], Veterans Administration Hospital, Iowa City, Iowa 52242, USA

Abstract. The continuous retinoblastoma cell line Y79, grown in suspension culture, has been examined by transmission and scanning electron microscopy, including freeze-fracture replica preparations. Four classes of cells were distinguishable by their size and surface characteristics. The surface structures included blebs, filopodia, lamellipodia, microvilli, and microplicae. The possible origin of a most unusual type of cell, apparently forming natural clones, is discussed.

Zusammenfassung. Die kontinuierliche Retinoblastom-Zellinie Y79, die in einer Suspensionskultur gezüchtet wurde, wurde untersucht mit Transmissions- und Rasterelektronenmikroskopie einschließlich Gefrierbruchpräparationen.

4 Klassen von Zellen waren zu unterscheiden aufgrund ihrer Größe und Oberflächenbeschaffenheit. Die Oberflächenstrukturen enthalten Blasen, Filopodien, Lamellipodien, Mikrovilli und Microplicae.

Die mögliche Herkunft eines recht ungewöhnlichen Zelltyps, der offenbar natürliche Klone produziert, wird diskutiert.

Introduction

Retinoblastoma is a tumor with several unusual features. In about 45% of patients, it is an autosomal dominant disorder with almost 100% penetrance. Deletion of a small segment of chromosome 13 has been recognized recently in several patients. Extensive necrosis is a common histologic feature, and spontaneous regression is observed in 1%–2% of cases.

Studies of the ultrastructural characteristics of retinoblastoma cells (Ts'o et al., 1969; Albert et al., 1970; Reid et al., 1974) suggest that the cell of origin

Address for offprint requests: E.S. Meek, Department of Ophthalmology, University of Iowa, Iowa City, Iowa 52242, USA

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may be a multipotential embryonic retinal cell with the capability of differentiating in the direction of either photoreceptor or glial cells.

The potential for investigation of this tumor has been greatly enhanced by the introduction of two continuous cell lines; these are the Y79 line of Reid et al. (1974) and the WER1-Rb1 line of McFall et al. (1977). In contrast to most other tumor cells, both of these cell lines grow readily as clusters of cells in suspension in the medium. This probably reflects a high net negative charge on the cell membrane (McFall et al., 1977). We have examined the cell surface of Y79 cells by scanning electron microscopy (SEM) and freeze-fracture techniques, in addition to using light microscopy and conventional thin-section transmission electron microscopy (TEM).

Materials and Methods

Cell Cultures

A starter culture of Y79 cells in its 15th passage was kindly provided by Dr. J. Fogh of the Sloan Kettering Institute, New York. Cultures were grown in Falcon plastic flasks (Falcon Plastics, Oxnard, California), using RPM1–1640 medium supplemented with 15% heat-inactivated calf serum (Reheis Chemical Co., Phoenix, Arizona) containing amphotericin B (0.25 mg/ml), penicillin (100 units/ml), and streptomycin (100 units/ml). This medium was sterilized by passage through a 200 nm Millipore filter. Cultures were incubated at 37° C in a humidified atmosphere of 95% air–5% CO_2 and examined daily. Cell counts were performed after treatment of suspensions with 0.02% EDTA in Ca- and Mg-free phosphate-buffered saline (PBS) at pH 7.4, and counted with a Coulter Model B Counter (Coulter Inc., Hialeah, Florida). Samples were collected at lag, exponential, and stationary phase growth intervals.

Light Microscopy (LM)

Cells were washed in the PBS, pelleted by gentle centrifugation, fixed in buffered neutral formalin (BNF), embedded in paraffin wax, sectioned at 5 μm, then stained with haematoxylin and eosin. Also, whole cell smears were prepared, fixed in absolute ethanol, and stained with haematoxylin and eosin.

Scanning Electron Microscopy (SEM)

Samples were washed twice in Hank's solution at pH 7.4, fixed in 2% glutaraldehyde in 0.1 M phosphate or 0.1 M cacodylate buffer containing 3% sucrose at pH 7.3 for at least 1 h. The sample was then washed with buffer, transferred to 2% osmium tetroxide in buffer, then rinsed with buffer, and dehydrated in graded ethanols (35%, 70%, 95%, 100%). At the 70% ethanol stage, the suspended cells were filtered onto a 5 μm Nuclepore membrane. Critical point drying was carried out using CO_2; the specimen was coated with carbon and palladium-gold 40:60 in a vacuum evaporator and examined in a Cambridge Stereoscan S4–10 electron microscope.

Freeze-Fracture Preparations

Cells were fixed in glutaraldehyde, rinsed in buffer as above, and transferred to 25% glycerol. A thick paste of the cells was then loaded into a mirror-replica specimen holder, frozen in liquid Freon-22, and transferred to liquid nitrogen. The specimen was then fractured and a replica prepared in a Balzer's instrument model BAF-301. The replicas were cleaned with Chlorox and distilled water, then examined by TEM.

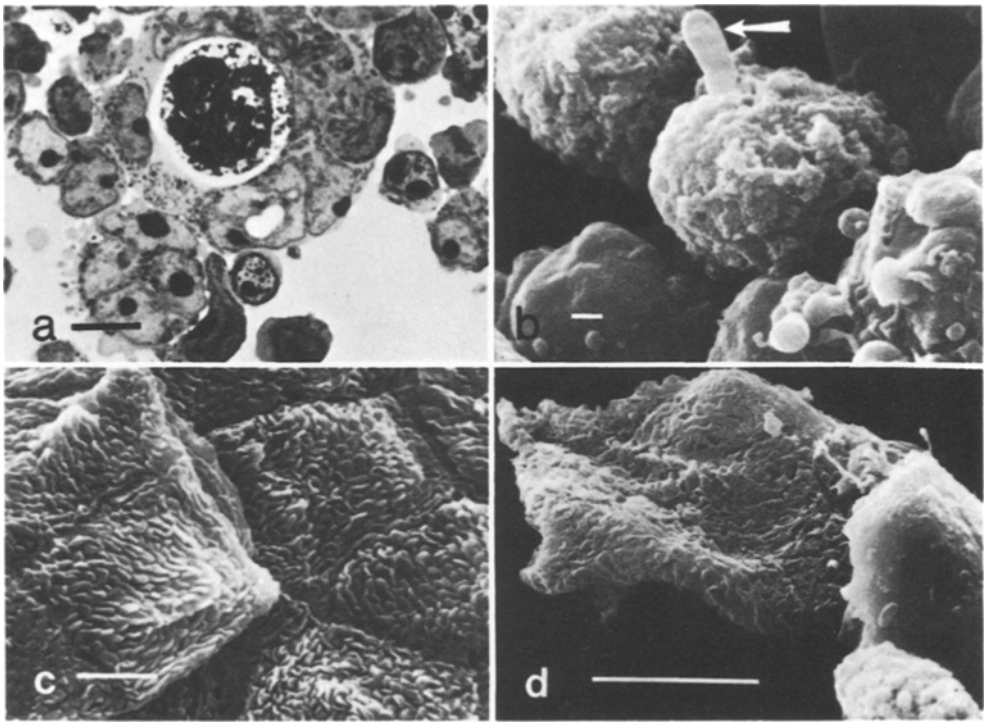

Fig. 1. a Suspended aggregate of Y79 cells; 1 μm thick section; toluidine blue; (bar is 10 μm). **b** Smooth and bleb-covered cell, lamellipodia (arrow); (bar is 1 μm). **c** Aggregate of microplicated cells type C; (bar is 5 μm). **d** Type D cell (top) compared with types A and B; (bar is 5 μm)

Transmission Electron Microscopy (TEM)

Procedures identical to those described for SEM through the 70% ethanol stage were carried out except that after the osmium rinse, cells were treated for 2 h in 0.5% urnayl acetate in 0.05 M sodium hydrogen maleate buffer pH 5.0 at 4° C in the dark. The cell pellet was dehydrated with ethanol, transferred to propylene oxide, infiltrated with Epon 812-propylene oxide 1:1, then embedded in Epon, and cured at 60° C for 48 h. Ultrathin sections were cut and stained with bismuth subnitrate or lead citrate for examination in a Siemens Elmiskop 101. A ±45° goniometer was used to examine the cells for junctional complexes. Semi-thin sections (1 μm) were also cut and stained with toluidine blue for examination by light microscopy.

Results

1. Light Microscopy

The majority of cells was small and undifferentiated with scanty cytoplasm and hyperchromatic nuclei; a second small population of larger cells with a relatively increased amount of cytoplasm was observed. A few cells were connected in short chains or ring formations. There were a substantial number of clumps of apparently viable cells surrounding a central mass of necrotic cell material (Fig. 1a).

Scanning Electron Microscopy

Most of the cells were aggregated in masses of up to 30 cells, but single cells were also observed occasionally. Three types (A, B, C) were seen, each apparently representing a different cell population. A rare fourth type (D) may have been a variant or transitional type of one of the three.

Of the total cell population, most were small (7–8 nm in diameter) and pseudo-spherical (type A). The surface morphology varied from cell to cell. Some were almost entirely smooth, others had irregularities covering part or almost all the surface (Fig. 1b). Blebs, ranging in size from 0.1 μm to 2.0 μm in diameter and differing in number from cell to cell, were commonly seen and were distributed irregularly. Some blebs were attached to the cell surface by long thin processes (filopodia), but not all filopodia were attached to blebs. Microvilli (0.1 μm in diameter, up to 5 μm long) were seen occasionally, and also a few lamellipodia.

A second type of cell (B), much less common, was about 10–12 μm in diameter. The surface morphology included the same features as described for the small type of cell (A). Some were smooth, many showed blebs, and there was a moderate number of filopodia. Microvilli were occasionally present; lamellipodia were rarely seen. The only external difference between these two populations was that of cell size.

A third type (C) (Fig. 1c), 15 to 17 nm in diameter, arranged in aggregates, was strikingly different. These were rarely encountered, their frequency in the total cell population being considerably less than 1 in 1,000. All the cells seen in these clusters showed the same surface morphology, giving the impression of a natural clone. They were polygonal in shape and their whole surface was covered with microplicae. After an extensive search, a very few single cells of this type were seen. Their general appearance was of a polygonal cell, with regularity of size, shape, surface features, and architectural arrangements within the cluster. The surface of each was entirely covered with microplicae about 300 nm in width and of variable height and length.

An exceptionally rare form of cell (D) is shown in Fig. 1d. These were flattened, single, and about 25 nm in diameter. The surface was generally rugose, with a few areas showing distinct microplicae; at the flattened edges these were seen as ruffles. Blebs were rarely seen and were very small; microvilli were not present.

Discussion

The cell membrane features of Y79 cells have been studied by TEM and SEM, using ultrathin sectioning and freeze fracture replica techniques as well as SEM.

Ultrathin sections showed the same general features described by other investigators (Reid et al., 1974; McFall et al., 1977). By TEM, most of the cells were small (type A). In many, the surface membrane was relatively smooth (Fig. 1b and d) and showed only a few, or small, protuberances on the surface. In the cells with a smooth exterior, few mitochondria were present in the cyto-

plasm. From this observation, it is suggested that they may be relatively inactive in a metabolic sense, although the internal structure does not suggest degeneration.

In others, the surface showed numerous convolutions which, judging by their size, corresponded to the blebs seen by SEM (Fig. 1 b). These protuberances contained ribosomes and some also included mitochrondria. There is no evidence of degenerative changes within the projections, and the position of mitochondria immediately below the cell membrane suggests a proximate source of energy for membrane activities.

SEM shows considerable variation in the number and size of blebs on the surface of the cells. McFall et al. (1977) noted in their SEM study that blebs were most numerous on the surfaces of both Y79 and WER1-Rb1 cells immediately after seeding and again at recurrent intervals of a few days. They remarked, however, that blebs and microvilli seemed to be as frequent on adherent cells as on suspension cells.

After investigating the consecutive stages of attachment of human diploid fibroblast cells to a surface, Rajaraman et al. (1974) reported that blebs became smaller and apparently withdrew during the development of filopodia. They suggested that blebs may contain the precursors of microfilaments and that such structual formations may be a response to trypsinization.

Time lapse cinephotomicrography of a transformed hamster cell line by Puck (1977) showed blebs in a state of constant agitation, executing oscillatory movements. Transformed cells in vitro can reacquire the morphologic and structural characteristics of normal cells by raising the intracellular level of cyclic adenosine 3',5'-monophosphate (cAMP) (Puck, 1977). This alteration is associated with change in the pattern of microtubules and microfilaments from the disorganized state seen in malignant cells to the organized pattern that appears to be characteristic of the particular state of differentiation. The possibility that the blebs of the Y79 cells depend on microfilament and microtubule organization, and that cyclic nucleotide levels control the activity of these structural elements, is currently receiving our attention.

By TEM, the surface of many cells is extremely convoluted (Fig. 2a). In such cases, the area of cell membrane in contact with the surrounding medium is unusually extensive in relation to the cell volume.

A very small number of cell clusters were seen to be of the distinctive microplicated type shown in Fig. 1c. The similarity of the cells within a cluster suggests that these may have developed as a natural clone. After considerable search, a few isolated single cells with this type of surface morphology were found. Their general external appearance suggests a differentiated polygonal epithelial type of cell; the surface of these cells is entirely covered with microplicae.

The nature of these microplicated cells is not known. Efforts to establish clones for the propagation of large numbers have been thwarted by their extreme paucity in the mixed cultures. Cells of this type could not be identified with certainty in ultrathin sections so that their internal structure has not been defined. The possibility that these are simply normal cells carried over from the original clinical specimen has been considered. Prolonged search of a three-day-old

culture of retinoblastoma cells from a tumor removed from a two-year-old boy revealed a very small number of these cells. In the case of Y79 cells, however, it seems unlikely that admixed normal cells could be propagated over long periods of time since normal cells are known to have a limited reproduction span in vitro, and nonreplicating cells would be diluted to vanishing point during repeated subculturing. Moreover, these cells are apparently growing in suspension in the same manner as the other Y79 cell types, a feature that reflects a high net negative surface charge (McFall et al., 1978) and is more characteristic of malignant rather than non-malignant cells. Microplicae have been seen on other types of cells: for example, a study of intestinal epithelial cells showed plicae on the lateral surfaces (Vial and Porter, 1975), but these were considerably larger in area although thinner (0.1 μm thick; 1–4 μm in length).

Reports by Yoneda and van Herick (1963) and Huang et al. (1970) describe the presence of polygonal cells in some cultures as well as spontaneous changes in the preponderance of different morphological cell types from time to time. The cell shown in Fig. 1d (type D) may represent a transitional stage between the microplicated type (Fig. 1c) and some other form. In this example, there is variation over the surface in the size and distribution of microplicae. At the edges of the cell these are seen as ruffles similar to those described by Rajamaran et al. (1974) in human diploid fibroblasts and thought by them to represent a means of locomotion. Marginal ruffles have also been seen in transformed cells in vitro (Porter et al., 1973).

Since these microplicated cells may have differentiated from the small undifferentiated cells, attempts were made to influence the morphology of Y79 cells by exposure to cyclic adenosine 3',5'-monophosphate (cAMP) and its more soluble analogue dibutyryl cAMP (DBcAMP). These substances have been shown to alter the morphologic and biochemical characteristics of several different types of experimental tumor cells toward a more normal pattern (Johnson, 1975; Prasad et al., 1975; Breckenridge, 1975), but preliminary attempts with Y79 cells have not resulted in any noticeable morphologic change in cell types so far.

Ohnishi (1977) proferred a model for the genesis of Wintersteiner-Flexner rosettes, beginning with the development of terminal bars and arrangements of the cells into a Wright rosette (that lacks a lumen), and progressing to the appearance of a central lumen with the Wintersteiner-Flexner rosette. Ts'o et al. (1969) also demonstrated joining terminal bars in rosette and floret cells, and remarked on the resemblance to the external limiting membranes of the normal retina. In screening Y79 cultures for rosette formation by light microscopy, it was observed that many of the cell aggregates showed, on section, a necrotic mass in the center (Fig. 1a). It is thought that with repeated cell division and increase in the number of cells present in a cluster of suspension cells, the central cells are deprived of the facility for rapid interchange of nutrients and metabolites with the surrounding medium, and so undergo degeneration and necrosis. Possibly this debris may be absorbed in the course of time, in which event the final structure might resemble a rosette. It is unlikely, however, that the phenomenon shown here pertains to the usual mode of rosette

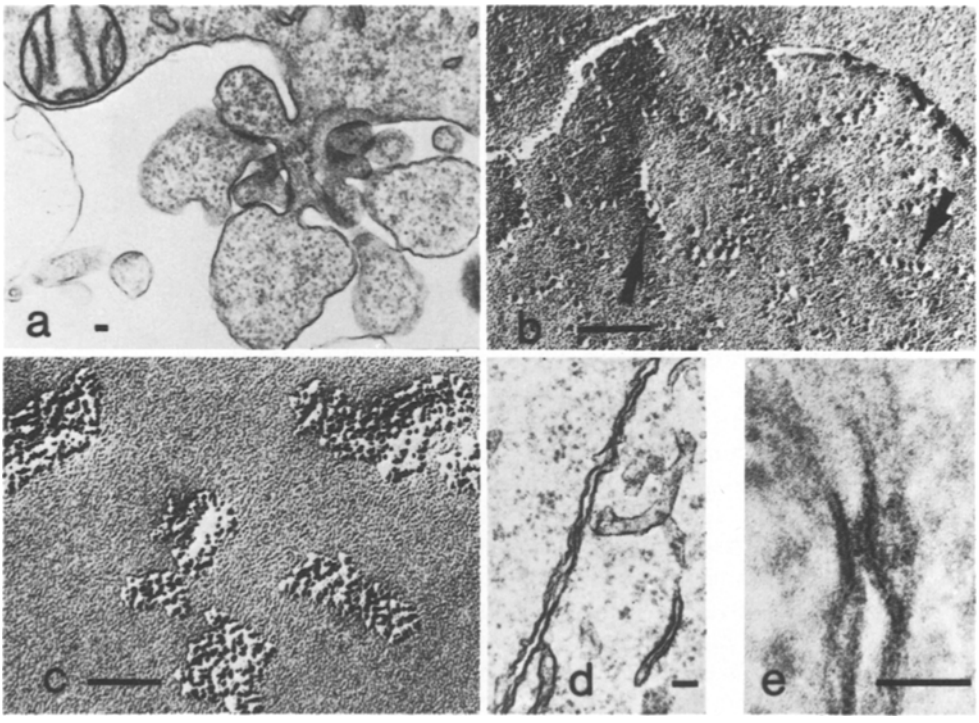

Fig. 2. a Cell with elaborately convoluted surface, bar is 0.1 μm. **b** Freeze-fracture replica showing diffusely scattered particles and some linear aggregates (arrows) on cell membrane; (bar is 0.1 μm). **c** freeze-fracture replica showing particle aggregates; (bar is 0.1 μm). **d** Section showing closest apposition of cell membranes; (bar is 0.1 μm). **e** Higher magnification; (bar is 50 nm)

formation; it probably results coincidentally from the particular steric arrangement of the cells in culture. These degenerative changes, together with the frequent observation of necrosis within the tumors in vivo, may suggest that retinoblastoma cells are unusually susceptible to restriction of nutrient supply, a condition likely to be associated with a high level of metabolism in an embryonic type of cell.

Examination of the cells by the freeze-fracture technique revealed the presence of discrete structures on the cell membranes. Gap or tight junctions could not be positively identified by the freeze-fracture technique, although some linear arrangements within fields of diffusely scattered particles could be interpreted as the early stages of development of either of these intercellular junctions (Fig. 2b).

Gap junctions are described as 'aggregates of intermembranous particles in two opposed membranes meeting particle to particle' (Larsen, 1977). They are thought to be sites for intercellular communication by means of molecular signals. The particles of gap junctions consist of subunits that aggregate to form a hexagonal cylinder, about 7 nm in diameter, with a central channel (Staehelin and Hull, 1978). The particles seen in the Y79 cells vary slightly in size but average 10–11 nm in diameter.

The linear network of mature tight junctions was not found nor were particles found in the parallel rectilinear arrays demonstrated by Hanna et al. (1976) to be often present in the vicinity of gap junctions on the membranes of glial cells. By thin section, close opposition of cell membranes (as close as 4.4 nm) was seen but the characteristic pentalaminar structure of tight junctions could not be unequivocally demonstrated (Fig. 2d). This distance of separation is appropriate for gap junctions (Sandri et al., 1977). The distribution of particle aggregates within a field free of other particles (Fig. 2c) is characteristic of Gray Type II postsynaptic complexes (Sandri et al., 1977).

Desmosomes have not been seen in our preparations. Although junctional deficiencies are common in neoplasms (Weinstein et al., 1976) the contribution, if any, of these defects to the properties of invasiveness and ability to metastasize has not been determined. The fragility of Y79 cell membranes has been remarked on by Reid et al. (1974).

The elaborate convolutions of the nuclear membrane in retinoblastoma cells have been remarked upon by many authors. Nuclear evaginations into cytoplasm and cytoplasmic invaginations of the nucleus are common. Popoff and Ellsworth (1969) described the prominent margination of chromatin in these cells as a triple-membered structure (TMS) sometimes enveloped by the nuclear envelope. These authors consider it to be a pathological exaggeration of a process that occurs in normal retinoblasts during maturation. Single TMS often line the inner perimeter of the nucleus and terminate at the nuclear pores. We examined the nuclear membrane by freeze-fracture technique but did not observe any abnormalities in the nuclear pores themselves. The complex convolutions of the nuclear membrane undoubtedly increase the surface area of this structure, and this may signify a need for unusually rapid exchange of material between nucleus and cytoplasm.

The junctional structures of the cell membrane have proved to be difficult to classify with certainty. This may reflect abnormalities in the synthesis and assembly of the membrane components, which may be related in turn to the behavior of these cells. Since the microtubule and microfilament systems play a role in membrane morphology, a study of these structures and their response to cyclic nucleotides is in progress.

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