Establishment of Cell Lines of Uveal Melanoma

Methodology and Characteristics

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Six continuous cell lines have been established from choroidal and ciliary body melanomas. These lines have been maintained in culture for at least 100 in vitro population doublings for periods over 1 year. They were established initially using a human diploid fibroblast strain MRC-5 as a feeder layer. Cells were grown in Ham's F-12 medium containing fetal bovine and horse sera and supplemented with glucose, cholera toxin, and epidermal growth factor. Culture doubling times ranged from 72-96 hr; cloning efficiencies ranged from 1-5% in the absence of a feeder layer. Six cell lines were studied in detail by electron microscopy, and all were found to have evidence of melanosomes and/or premelanosomes. The morphology of the cells was characteristic of melanomas as defined by the Callender classification, with cell types ranging from spindle A to epithelioid. Karyotypic studies revealed the presence of only human chromosomes with modal numbers ranging from 48-54 in the different lines. Invest Ophthalmol Vis Sci 25:1284-1299, 1984

Malignant melanoma is the most common primary malignancy of the eye. It occurs more frequently in the uveal tract of the eye than in any other site in the body except the skin. Treatment of this tumor has become the topic of intense controversy. This is the result of data from the Armed Forces Institute of Pathology supporting the hypothesis that, in the past, enucleation of the melanoma-containing eyes was an important causative factor in the metastasis of this tumor.

A lack of any culture system that permits the routine in vitro growth of uveal melanomas has hindered the study of basic properties of these neoplastic cells. We now report six uveal melanoma cell lines, which have been maintained in culture beyond 100 in vitro population doublings for periods over 1 year and that we believe are established in vitro. We describe the techniques used to establish these cell lines and compare their morphologic features to those established for uveal tissues studied in vivo.

Materials and Methods

Culture Methods

Tumor-containing eyes were received within 24 hr following enucleation and were rinsed three times in sterile Hanks balanced salt solution (HBSS). Pupil-optic nerve sections of the eyes were cut, and the specimens of the tumor remaining in the calottes were minced into 1 mm³ pieces. The minced tissue was stored at room temperature in complete growth media until being placed onto feeder layers.

Feeder Layers

The human diploid fibroblast strain, MRC-5, was used as a feeder layer for growing the melanoma tissue. MRC-5 cells that had achieved less than 20 mean population doublings in vitro were inoculated into 100-mm diameter petri dishes (Falcon; Oxnard, CA) and grown to confluency. Immediately prior to placing the tumor tissue onto the fibroblast monolayer, the confluent MRC-5 cultures were rinsed with HBSS lacking phenol red and irradiated for 10-15 sec at a distance of 22 inches from a General Electric #G-30T8 bulb, for an exposure of 125-130 ergs/cm² of ultraviolet (UV) light.

Plating of Tumor Tissue

Three 1 mm³ tumor pieces were placed into each petri dish containing a confluent, irradiated feeder layer. A number 10 Bard-Parker scalpel blade was used to attach each piece of tissue to the surface of the petri dish. The three tissue pieces were placed equidistant from each other on the feeder layer and pushed firmly into the plastic surface of the petri dish with the point of the scalpel blade. Each tissue slice was attached so that it would not float when the medium was added subsequently to the dish.
### Table 1. Summary of cases from which cell lines were derived

<table>
<thead>
<tr>
<th>Cell line no.</th>
<th>Eye path no.</th>
<th>Date of enucleation</th>
<th>Age, race, sex</th>
<th>Size of tumor (largest diameter at base x height in mm)</th>
<th>Histopathologic diagnosis of cell type</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLI65</td>
<td>E82-1607</td>
<td>9/2/82</td>
<td>32, W, M</td>
<td>20 x 15</td>
<td>mixed</td>
<td>presented with angle closure glaucoma</td>
</tr>
<tr>
<td>OM421</td>
<td>E82-2040</td>
<td>11/24/82</td>
<td>66, W, F</td>
<td>21 x 18</td>
<td>mixed</td>
<td>grossly amelanotic</td>
</tr>
<tr>
<td>OM431</td>
<td>E82-2160</td>
<td>12/14/82</td>
<td>44, W, M</td>
<td>17 x 10</td>
<td>epithelioid</td>
<td>orbital recur. subsequent exenteration</td>
</tr>
<tr>
<td>OM439</td>
<td>E83-79</td>
<td>1/13/83</td>
<td>78, W, F</td>
<td>15 x 14</td>
<td>mixed</td>
<td>invasion into scleral vein</td>
</tr>
<tr>
<td>OM443</td>
<td>E83-147</td>
<td>1/25/83</td>
<td>62, W, M</td>
<td>12 x 11</td>
<td>mixed</td>
<td>previous history lead poisoning</td>
</tr>
<tr>
<td>OM449</td>
<td>E83-199</td>
<td>2/2/83</td>
<td>75, W, F</td>
<td>17 x 6</td>
<td>mixed</td>
<td>heavily pigmented</td>
</tr>
</tbody>
</table>

#### Medium

The MRC-5 cells were grown and maintained in Eagle’s minimal essential medium (Flow Laboratories, Inglewood, CA) supplemented with 900 mg/ml glucose, 6.6 mg/ml sodium pyruvate, and fetal bovine serum (10%, v/v). For growing melanoma cells, Ham’s F-12 medium was supplemented with 2,000 mg/l

![Image](image_url)
glucose, $1 \times 10^{-9}$ M cholera toxin (Sigma Chemical Company; St. Louis, MO), 10 µg/ml epidermal growth factor (bovine, partially purified; Collaborative Research; Waltham, MA) fetal bovine serum (15%, v/v), and donor horse serum (5%, v/v). Empirical testing indicated that medium and serum containing greater than 1 ng/ml of endotoxin or adventitious bovine viruses inhibited the growth of the melanoma cells. Therefore, all medium and serum used in these studies contained less than 1 ng/ml of endotoxin and no detectable adventitious bovine viruses. All cultures were renewed with complete growth medium three times per week.

Growth of Melanoma Cells

Cultures were maintained at 36.5°C in a humidified incubator gassed with 5% CO$_2$. Cells could be seen migrating out of the tissue pieces 10-14 days following initial plating of the tumor tissue. Over a period of 4-8 weeks, the irradiated feeder layers would detach from the plastic surface and be replaced by melanoma cells. In some instances fibroblasts also would migrate from the tumor tissue, but these cells generally did not survive. After approximately 8 weeks in culture, when the melanoma cells covered 25-50% of the surface of the petri dish, and the feeder layer had begun to disappear, the cells could be transferred to new culture dishes. Trypsinization was performed using 0.05% trypsin (Difco; Detroit, MI; 1:250) with 0.5 mM Na$_2$/EDTA (Baker; Phillipsburg, NJ). Subsequent passages were made at each subsequent confluence.

Measurement of Culture Doubling Times

In order to determine the in vitro doubling time of the melanoma cells, 3-5 $\times 10^4$ cells were placed into 60-mm diameter petri dishes and counts performed on duplicate dishes every 24-72 hr. During the course of the growth experiments, the cultures were renewed with fresh medium every 72 hr.

Light Microscopy

The histopathologic study of the cells in culture was performed in several ways: (1) cells were examined and photographed as they grew in vitro; (2) smears

Fig. 2. Spindle cell melanoma cells seen through inverted microscope after 1 year in tissue culture and more than 100 doublings ($\times 200$).
were made of floating cells; (3) cells in tissue culture were scraped from the plastic and formed into a pellet by centrifugation (748 × g) for 5 min. Tissue culture material was fixed in 10% phosphate buffered formalin and sections were stained with hematoxylin and eosin.

Table 2. Morphology of cell types*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Principal cell lines seen on EM</th>
<th>Cytoplasmic filament</th>
<th>Mitochondria</th>
<th>Endoplasmic reticulum</th>
<th>Ribosomes</th>
<th>Golgi</th>
<th>Melanin granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL165</td>
<td>spindle A, spindle B</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>epithelioid</td>
<td>-</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>OM421</td>
<td>spindle A, spindle B</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>epithelioid</td>
<td>-</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>OM431</td>
<td>epithelioid</td>
<td>-</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>OM439</td>
<td>spindle A, spindle B</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>spindle B</td>
<td>-</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>OM443</td>
<td>spindle A, spindle B</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>spindle B</td>
<td>-</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>OM449</td>
<td>spindle A, spindle B</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Structures are indicated in a semi-quantitative manner; ++++ seen in profusion; +++ frequent; ++ occasional; + sparse; and - absent.
Electron Microscopy

Both the original tumors as well as cells from tissue culture were examined. Tumor material was minced into fine pieces. Cells in tumor culture were formed into a pellet as described above. Tumors and pellet were fixed in either Dalton's chrome osmium for 1 hr or 3% phosphate-buffered glutaraldehyde-sucrose for 1 hr followed by chrome osmium. Specimens were dehydrated in graded ethyl alcohol and embedded in Epon-Araldite mixture. Ultrathin sections were cut with an LKB microtome and double stained in uranyl acetate followed by lead citrate. Micrographs were taken with a JEOL electron microscope with an 80-kV accelerating voltage and a 50-μm objective aperture.

In addition, cell cultures were fixed as a monolayer in the flask, washed, and dehydrated as above. Following this, a thin layer of epon was poured into the flask and allowed to polymerize at 60°C for approx-
approximately 6 hr. Then the epon with the cells in it was removed from the flask and a sandwich made by pouring another layer of epon on the side where the cells were. After polymerization, small blocks were cut and ultrathin sections were prepared as above.

For scanning electron microscopy (SEM) following fixation, as above, the specimens were dehydrated through a graded series of alcohols and dried in CO₂ in a Samdri PVT-3 critical point drying apparatus. The specimens then were coated with gold palladium and examined with a JEOL scanning electron microscope.

Cytogenetic Technique

Chromosome studies were done on cultures in exponential growth. Fresh medium containing 20 mcg/ml of colcemid (Sigma) was added to subconfluent cultures. The cultures then were incubated at 36.5°C for 3-18 hr. The cells then were trypsinized,
suspended in 0.075 M KCl for 10 min, and fixed with methanol:acetic acid (3:1, v/v) at 4°C. For chromosome visualization, slides were made of the suspended cells and G-T-G (Giemsa-trypsin-Giemsa) staining performed to allow for analysis of metaphase cells.

Results

The pertinent clinical and pathologic findings regarding the tumors used for the establishment of cell lines are given in Table 1.

Growth and Appearance of Melanoma Cells in Tissue Culture

After 10–14 days following the initial plating of the tumor tissue, melanoma cells could be seen growing out from the explants. Over a period of 4–8 weeks, the irradiated feeder layer detached from the plastic surface and was replaced by melanoma cells. In some instances, fibroblasts also would migrate from the tumor tissue, but these cells did not survive. After approximately 8 weeks in culture, when the melanoma cells covered 25–50% of the surface of the petri dish, and the feeder layer had begun to disappear, the cells were transferred to new culture dishes.

On the basis of phase contrast microscopy, the cell shape could be determined easily. These fell within the spectrum of the Callender classification, and the cell outline could be determined. The nuclear detail could not be definitively distinguished by phase contrast examination, but on subsequent TEM examination (see below) nuclear characteristics, which are the most important criteria for differentiation of cell type, correlated with the cell shape in a manner consistent with the Callender classification. The appearance of the cells varied from predominantly epithelioid (OM431) (Fig. 1) to almost pure spindle (OM439, OM443, OM449) (Fig. 2). The remaining cultures (HL165 and OM421) were predominantly spindle-shaped with a small component of epithelioid cells (Fig. 3). Cell doubling time varied among the cultures from 72–96 hr.

Light Microscopic Appearance of Cell Lines

Cells of the six ocular melanoma cell lines, when examined by light microscopy, conformed to the cell types of the Callender classification: spindle A, spindle B, and epithelioid. The cell lines have become pro-
Fig. 7. A spindle B-type melanoma cell having a plump cell body and indentations of the nuclear membrane (arrow). Note the large centrally located nucleolus (N). In the cytoplasm there are mitochondria (M) and pigment granules (P).

progressively less pigmented with continuous passage, but with the Fontana stain, positive melanin granules can be identified in all of the cell lines.

Electron Microscopic Findings of Cultured Cells

The six cell lines have been examined by transmission electron microscopy: HL165, OM421, OM431, OM439, OM443, and OM449. Table 2 presents a summary of the ultrastructural findings of these lines.

On TEM examination, three cell lines contained only spindle A and spindle B type cells (OM443, OM439, and OM449); two cell lines contained spindle A and B as well as epithelioid cells (HL165 and OM421); and one line was composed of pure epithelioid cells (OM431). The appearance of various cell types was similar among the different cell lines.

The spindle A cells observed in HL165 displayed elongated cell bodies, small nucleoli, and chromatin at the margins of the nuclear membranes. The cytoplasm of these cells contained few mitochondria, some rough endoplasmic reticulum, and ribosomes.
Single and/or compound melanin granules were found and numerous 10 nm cytoplasmic filaments were seen (Figs. 4–6). The spindle B cells observed in line OM449 displayed plump cell bodies and invaginations of the nuclear envelope. The nuclei were somewhat larger than the spindle A type, but as in spindle A cells, chromatin was found at the periphery of the nuclear envelope. In the cytoplasm there were short branches of endoplasmic reticulum, mitochondria, lipid, Golgi apparatus, melanin granules, and numerous ribosomes (Figs. 7, 8).

Cells of the epithelioid type found in HL165 showed polygonal cell bodies. The nuclei displayed indentation of the nuclear envelopes, well-formed nucleoli, and diffuse deposition of chromatin throughout the nucleus. Microvilli were found projecting from the cell membranes (Fig. 9). The epithelioid cells seen in line OM431 showed well-formed nucleoli and their cytoplasm contained short branches of endoplasmic reticulum, mitochondria, melanin granules, premelanosomes, and numerous ribosomes (Figs. 10–12).

By scanning electron microscopy (SEM) in line
HL165, spindle and epithelioid cells were observed. The spindle cells had elongated dendritic-like processes (Fig. 13).

By the use of transmission electron microscopy, the presence of melanin granules in all cell types was confirmed by treating sections with potassium permanganate (KMnO₄), and bleaching the granules containing melanin (Fig. 14). In general, the ocular melanoma cells in vitro were similar in their ultrastructural appearance to previous descriptions of freshly fixed uveal melanoma tissue.

Cytogenetic Findings

It was found that all of the cell lines examined contained human chromosomes in diploid or near diploid numbers. Several types of chromosome abnormalities including breaks and rearrangements were noted that are being examined further.
Discussion

The first reported attempt to grow ocular melanoma cells in cell cultures appears to have been that of Kirby described in 1929.7

Kirby's studies, as well as subsequent observations of in vitro growth of uveal melanoma, consisted of relatively short-term experiments.7--18 It was demonstrated that uveal melanoma cells can survive in culture for periods up to several months or longer if transfer of the cells is not attempted. Although no continuous cell lines of uveal melanoma were established, some interesting features of this tumor were demonstrated. Irvine et al18 reported on cultures of five choroidal melanomas that "spontaneously transformed" from spindle cell to epithelioid type and vice versa. In subcultures they demonstrate intermediate forms between the two cells, suggesting a transition from epithelioid to spindle.

Since the 1950s, it has become apparent that there...
is no single universal growth medium, and that a particular cell type may grow well in one medium but not another. The culture medium that was shown to be optimal for the growth of uveal melanoma cells in the present study was a modification of Ham's F-12.\textsuperscript{19} The use of feeder layers to support cell attachment and growth also has been demonstrated to be helpful in growing cells in vitro with specific cell types required as substrates to promote the survival and growth of various tissues.\textsuperscript{20} For example, fibroblasts, retinal pigment epithelium,\textsuperscript{21} and rat smooth muscle cells\textsuperscript{22} will permit the routine growth of human retinoblastomas in vitro. It also has become apparent that cells in tissue culture require hormones and growth factors.\textsuperscript{23} The addition of serum alone is not always sufficient to provide these, however, and additional specific growth-modifying factors also may be needed.\textsuperscript{24} Additional factors such as pH of the culture medium, the particular mechanical or chemical methods used for cell dispersion, seeding density,
and incubation temperature also may play a critical role in the success of sustained cultures.\textsuperscript{25} In some tissues that represent a mixture of cell types, it is necessary to selectively inhibit certain cells. For example, in attempting to grow normal melanocytes from skin, one must suppress overgrowth by fibroblasts and keratinocytes; for this purpose corticosteroids and phorbol 12-myristate 13-acetate (PMA) have proved effective.\textsuperscript{25-27} In the successful establishment of nonocular animal melanomas and cutaneous melanomas, a number of growth promoting factors have been identified: melanocyte stimulating hormone (MSH), cyclic AMP, imidazole, dexamethasone, and others.\textsuperscript{28-32}

Over a 3-year period, we systematically studied the effects on uveal melanoma of a variety of media, feeder cell layers, and growth modifying factors. The present success in growing uveal melanoma cells
results from a combination of factors: (1) the use of Ham's F-12 medium; (2) use of human diploid fibroblast cells as a feeder layer, with the MRC-5 strain proving particularly effective; (3) the addition of epidermal growth factor; and (4) the addition of cholera toxin. Epidermal growth factor and cholera toxin appear to have a synergistic effect by inhibiting contaminating cells and stimulating proliferation of melanocytes on a supporting cell layer. Additional factors that facilitate development of long-term growth appear to be the combination of fetal bovine serum with donor horse serum. A high concentration of glucose (2,000 mg) also seemed to be effective in achieving long-term cultures.

We believe that the ability to establish cell lines of uveal melanomas will open the way for further morphologic, immunologic, and karyotypic studies, some of which have been initiated already. In addition, the
sensitivity of melanoma to various chemotherapeutic agents can be tested. Finally, these cell lines will be useful in the search for oncogenes in this tumor.

Key words: uveal melanoma, cell culture, cell line, in vitro

References


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