Effect of Melanocyte Stimulating Hormone on Human Cultured Choroidal Melanocytes, Uveal Melanoma Cells, and Retinal Epithelial Cells

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Purpose. To establish methodology for the culture of human choroidal melanocytes to compare their responsiveness to melanocyte stimulating hormone (MSH) with that of their transformed melanoma counterparts and with that of the retinal epithelial cell.

Methods. Choroidal melanocytes from the choroid of eyes enucleated for the presence of malignant melanoma were cultured in MCDB 153 medium supplemented with insulin, transferrin, hydrocortisone, glutamine, nystatin, vitamin E, phorbol myristate acetate, bovine hypothalamic extract, cholera toxin, and chelexed fetal calf serum.

Results. High yields of pure spindle-shaped bipolar melanocytes were obtained with a doubling time of 3 to 4 days in nine consecutive eyes. Cells continued to divide after 4 months in culture. In contrast, uveal malignant melanoma cells grew rapidly in a relatively simple medium of Ham's F12:DMEM (1:1) supplemented with fetal calf serum, insulin, transferrin and glutamine. This medium was unable to support choroidal melanocytes. Choroidal melanocytes were DOPA-positive with appreciable tyrosinase activity that significantly increased with treatment with MSH. MSH also significantly altered the size, local density, and distribution of primary and mature melanosomes of ocular melanocytes. In contrast, uveal melanoma cells had a low level of tyrosinase activity and failed to respond to MSH with either an increase in enzyme activity or melanosome size. Retinal epithelial cells failed to show significant tyrosinase activity under the conditions studied or any increase in melanosome size in response to MSH.

Conclusion. Ocular melanocytes show evidence of regulation by MSH and a range of mitogenic stimuli unlike the transformed melanoma cells, implying a loss of regulatory control in the latter. Invest Ophthalmol Vis Sci. 1994; 35:826-837.

Malignant melanomas of the posterior uveal tract (ciliary body and choroid) are the most common primary intraocular malignancy in adults. Although uncommon, they represent approximately 80% of all noncutaneous melanomas.1 These tumors pose a serious threat to life, and approximately half the patients die from the disease within 15 years.2 Despite recent advances in treatment, the prognosis for patients with these tumors remains unaltered.2,3

Comparative studies of how the normal skin melanocyte differs from the cutaneous malignant melanoma cell have proved important in understanding factors involved in progression and metastasis of cutaneous melanoma.4-6 Similarly, there is a need to understand how ocular uveal malignant melanoma cells differ from their normal precursor cell, the uveal melanocyte, but as yet there has been little comparative work largely because of the difficulties in culturing ocular melanocytes. During the last 8 years, four groups have reported on the successful culture of ocular melanocytes.7-11 In this study, we aimed to establish reproducible methodology for the culture of normal choroidal melanocytes and then to compare unstimulated and MSH-stimulated melanogenic activity of ocular melanocytes.

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826
lar melanocytes with that of their transformed counterparts, uveal melanomas, and with that of the other pigmented cells seen in the eye, the retinal pigment epithelial cell. Additionally, for comparison we have included human cutaneous melanocytes and mouse murine melanoma cells.

In culturing choroidal melanocytes, we have followed methodologies that are well established for skin melanocyte culture in many laboratories. Although there are several different regimes in use that result in production of melanocytes proliferating to various degrees, it is well accepted that cutaneous melanocytes will not survive in culture unless stimulated with two classes of mitogens, one of which stimulates the cyclic AMP drive and the other, the protein kinase C or tyrosine kinase drive. In vivo, it is thought that the tyrosine kinase drive will be stimulated by bFGF produced by keratinocytes in contact with melanocytes. Some groups have also found serum to be important to the growth of skin melanocytes in vitro. A recent study investigating the growth requirements of cultured human uveal melanocytes has confirmed that for uveal melanocytes, as for skin melanocytes, bFGF or TPA plus a cyclic AMP stimulator and serum appear to be required for growth of these melanocytes. In the current study, in line with this report and with our previous work on skin melanocytes, we have used a medium containing TPA, cholera toxin, and hypothalamic extract (containing bFGF) that we have previously found to be successful for the culture of skin melanocytes.

Using this medium, we have successfully cultured choroidal melanocytes and begun to compare their differentiated behavior with that of their transformed counterparts, the uveal melanoma and with that of the pigmented retinal epithelial cell.

MATERIALS AND METHODS

Tissue Samples

We took samples of ocular tissue from freshly enucleated eyes which were removed because of the presence of large posterior uveal melanoma tumors. Immediately after enucleation, and under sterile conditions, the globe was opened along an axis parallel to the longest diameter of the tumor and a calotte containing normal retina and choroid was removed. When tumor samples were taken from the globe, we took care to ensure that this did not compromise the histologic typing and staging of the tumor, for ethical reasons. We dissected the tumor sample and placed one part immediately in liquid nitrogen for analysis of tyrosinase activity, and the remainder in transport medium (1:1 of Ham’s F12: DMEM containing 10% fetal calf serum) for cell culture. The globe, with its residual tumor, was fixed in 3% buffered gluteraldehyde solution and processed for routine histological examination. We classified tumors according to the Armed Forces Institute for Pathology (AFIP) Classification of Melanocytic Tumors of the Uvea.

The overlying neuroretina was removed from the calotte and discarded. Bruch’s membrane and remnants of retinal pigment epithelium and inner choroid were detached and placed in transport medium to form the “inner choroid” preparation. The remaining sclera from the calotte, with attached outer choroid, was treated similarly to form the “outer choroid” preparation.

We obtained normal human skin samples from surgical specimens removed during cosmetic surgery (abdomen, breast and thigh), or circumcision (fore-skim). At the time of excision the skin was wrapped in sterile gauze, soaked in normal saline, and placed in a sterile container. Skin samples could be stored at 4°C for 24 hours without any detectable loss of cellular viability.

(In all studies involving human material, the tenets of the Declaration of Helsinki were followed and none of these studies affected the clinical treatment of the patients or influenced the choice of treatment).

Preparation of Bovine Hypothalamic Extract

We made bovine hypothalamic extract (BHE), to be used in culture of choroidal melanocytes, by homogenizing 64 g of hypothalamic tissue (freshly obtained from a local abattoir) in 64 ml of ice cold 0.1 M NaCl for 3 minutes in a Waring blender. The homogenate was extracted with stirring at 4°C for 2 hours, and centrifuged at 10,000 g for 30 minutes. The supernatant was decanted and the pellet was re-extracted overnight. The second extract was centrifuged as before and the supernatants combined. Lipid was removed using streptomycin sulphate (5 g/kg tissue in 50 ml H2O, pH 8.0) overnight. The extract was then centrifuged at 20,000 g for 30 minutes. After determining the protein concentration by the Lowry method, the pellet was discarded and the supernatant divided into 100 mg aliquots, and frozen at −20°C. The aliquots were thawed as needed and filter sterilized.

(All biochemical reagents were purchased from Sigma, U.K. unless stated otherwise).

Isolation and Culture of Choroidal Melanocytes

The inner and outer choroid preparations were removed from the transport medium, rinsed in Dulbecco’s phosphate buffered saline (PBS, Gibco BRL), and placed in a 34 mm diameter petri dish. Melanocytes from the outer choroid preparation were liberated from the sclera by gentle scraping with curved forceps and the inner choroidal preparation was...
chopped finely; in both cases this was followed by a type IA collagenase digestion (200 U/ml for 30 minutes at 37°C). Culture medium containing 10% fetal calf serum (FCS) was then added to the digest, and the tissue suspension was dissociated further by passage of the sample through a 19 g needle. The resulting cells were centrifuged at 250 g for 5 minutes and the pellet was resuspended in 25 cm² tissue culture flasks (Costar, U.K.) in MCDB153 culture medium supplemented with bovine insulin (10 µg/ml), human transferrin (10 µg/ml), hydrocortisone (2.8 µg/ml, Calbiochem), glutamine (2 mM, Gibco BRL), nystatin (10 U/ml, Gibco BRL), vitamin E (1 ng/ml), phorbol myristate acetate (PMA; 10 nM), cholera toxin (100 ng/ml), bovine hypothalamic extract (100 µg/ml), prepared as described above and chelexed fetal calf serum (2%, Advanced Protein Products). The cells were allowed to attach for 48 hours, at 37°C and 5% CO₂, before changing the media.

**Culture of Uveal Melanoma Cells**

We cut tumor samples into small cubes (<1 mm) with scalpel blades, digested them in type IA collagenase for 30 minutes in PBS, and passaged them through a 19 g needle. The resulting cell suspension was washed with DMEM culture medium supplemented with 10% FCS, centrifuged at 250 g for 5 minutes, resuspended and plated out in Ham’s F12: Dulbecco’s modified Eagle’s medium (1:1) supplemented with 10% FCS, insulin (10 µg/ml) and glutamine (2 mM, Gibco BRL).

**Isolation of Retinal Pigment Epithelial Cells**

We removed and dissected the anterior part of the globe from a line 5 mm posterior to the limbus, and then removed the neuroretina, after rinsing with PBS. The posterior globe was digested for 30 minutes in 0.2% trypsin in PBS. The release of the cells was aided by gentle pipetting. The digestion was stopped by the addition of medium containing 10% FCS. After washing with PBS, the cells were plated out in Eagle’s minimum essential medium supplemented with 20% FCS, glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml).

**Isolation of Skin Melanocytes**

We isolated skin melanocytes from tissue samples of both neonatal foreskins and adult skin (obtained from abdominoplasties and breast reductions) using the method described by Friedmann et al. Skin was cut into 1 cm squares after removing excess connective tissue, and incubated overnight in dispase (2 mg/ml, Boehringer Mannheim, Germany) at 4°C. The epidermis was separated from the dermis using fine forceps and incubated in 0.25% trypsin at 37°C for 5 to 10 minutes. After centrifugation, primary cultures were established in MCDB 153 medium containing genetical (100 µg/ml). After 2 to 3 days the medium was replaced with one without geneticin. The liberated melanocytes were cultured under the same conditions as described for the normal choroidal melanocytes.

**Tyrosinase Assay**

We plated out cells at densities of 6000 to 8000 cells per well in 96 well tissue culture plates. After 4 to 6 hours of cell attachment and growth, melanocyte stimulating hormone (MSH) was added to treatment groups as appropriate and the cells were incubated for between 24 to 48 hours before tyrosinase assay. We modified the tyrosinase assay of Jara for use in 96 well tissue culture plates to reduce the numbers of cells required. After the growth phase the media was removed from the wells and the cells were rinsed three times with 200 µl of PBS. The cells were then lysed by the addition of 45 µl of 0.1% Tween 20 in 0.1 M phosphate buffer (pH 6.8) and frozen at −80°C for 30 minutes. We then thawed and preincubated the plate at 37°C before starting the assay. For each plate the light absorbance at 490 nm was measured using a Dynatech plate reader, and the reaction was initiated by addition of 5 µl of 10 mM L-3,4 dihydroxyphenylalanine (L-dopa, Fluka UK) in 0.1 M phosphate buffer (pH 6.8). The plates were incubated at 37°C in the dark and the absorbance at 490 nm measured at timed intervals for up to 8 hours. The length of incubation depended on the enzyme activity present; in cells with appreciable tyrosinase activity 90 minutes was usually sufficient for calculation of the rate of tyrosinase activity, whereas in cells with little activity the enzyme reaction was allowed to proceed for up to 8 hours.

Tyrosinase activity has to be calculated and corrected for protein. Protein concentration was determined using the Bradford reaction²⁰ within the wells used for the tyrosinase assay. After the tyrosinase assay the plates were read at 630 nm before and after addition of 150 µl of Bradford reagent to each well. Change in absorbance was then calculated by subtraction and compared with bovine serum albumin standards to give the protein concentration.

**Dopa Staining**

We stained cultured melanocytes using the DOPA reaction for tyrosinase as described in.²¹ Cells were rinsed twice in PBS, fixed for 20 minutes in 4% formaldehyde solution in PBS, washed three times with PBS and incubated at 37°C for up to a 18 hours in the dark with 10 mM L-dopa (Fluka). After incubation, the cells were rinsed with distilled water, and fixed for 20 minutes in 4% formaldehyde and counterstained using Lillie’s Azure A-Eosin B method.²² Additional cultures of all cells were also incubated with 10 mM D-dopa (Fluka) as a non-metabolizable substrate and also with 10 mM L-dopa in the presence of the tyrosinase inhibi-
Effect of MSH 829

tor, phenylthiourea at 1 mM under identical conditions as controls for the dopa reaction.

Electron Microscopy

We fixed cell cultures with 3% gluteraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) and processed them into Agar 100 resin, in situ within tissue wells. Ultra thin sections were stained in alcoholic saturated uranyl acetate (5 minutes) followed by Reynold's lead citrate (2 minutes), and viewed on a Philips EM 400 transmission electron microscope.

We addressed the problem of true random sampling of melanocytes for melanosome quantification by allowing one worker (MF) to select and photograph cells, without knowledge of the source of cells or their treatment group. In practice, there were few differences in cell morphology and melanosome numbers between cells in individual cultures. Separate workers (MAP, TG), measured the longest diameter of melanosomes in individual random electron micrographs (magnification X 12,000) in ignorance of treatment group of the cells.

Statistical Analysis

Melanosome size was compared using Student's t-test with P < 0.05 as the level of significance. Data was demonstrated to have a normal distribution before carrying out any analysis.

RESULTS

Isolation of Choroidal Melanocytes

Electron microscopy of the intact original “inner” and “outer” choroidal preparations demonstrated the presence of choroidal melanocytes, with variable loss of RPE cells from the inner choroidal preparation as a result of removal of neuroretina as illustrated in Fig 1.

We established cultures of ocular melanocytes from both inner and outer choroidal preparations. In cultures from the inner choroidal preparation, large numbers of contaminating RPE cells were present, but cultures of ocular melanocytes from the outer choroid preparation consisted largely of melanocytes with approximately 5 to 25% RPE cells. In culture, melanocytes were easily distinguished from RPE cells on morphological grounds alone.

In culture, normal choroidal melanocytes (see Figs. 2b and c) are superficially similar to skin melanocytes (see Fig. 2a) and are usually bipolar and spindle-shaped with less than 1% of choroidal melanocytes multipolar (see Fig. 2d). Normal choroidal melanocytes (from either the inner or outer choroid) were plated out in T25 flasks at approximately 10% confluence. Under the prevailing culture conditions cells became 80 to 90% confluent over 3 to 4 weeks, by which time the percentage of contaminating RPE cells was 10% or less in the outer choroidal preparation and

FIGURE 1. Electron micrograph of the “inner choroidal” preparation reveals, in some areas (a), attached retinal pigment epithelial cells (RPE), although often they are missing (b). Beneath Bruch’s membrane (arrows), in the choroid, there are choroidal melanocytes (cm), occasional melanophages (m), and choroidal capillaries (cc) (space bar represents 1 μm).
around 25% in preparations of inner choroid. At this stage (3 to 4 weeks) cells were passaged, and by 6 weeks the cultures from the outer choroid contained more than 95% melanocytes. At the same stage, cultures from the inner choroidal preparation contained approximately 75% melanocytes, and RPE cells were progressively lost with time in culture. Cultures of around 90 to 95% pure melanocytes took on average 8 to 12 weeks to achieve when culturing from the inner choroidal preparation.
Comparison of Culture Conditions of Ocular Melanocytes, Melanoma Cells, RPE Cells, and Skin Melanocytes

To date we have established successful proliferative choroidal melanocyte cultures from 9 consecutive eyes. Ocular melanocytes were found to have the ability to attain a higher density in culture (see Fig. 2e) than equivalent cultures of skin melanocytes (as shown for melanocytes derived from neonatal foreskin in Fig. 2a). In our experience, melanocytes from neonatal foreskin grew to a higher density than those derived from adult skin but neither approached the high densities achieved by the ocular melanocytes in the same culture system. At the time of writing ocular melanocytes are surviving and proliferating after 6 months in culture. During the initial phase of this study several attempts to culture choroidal melanocytes were made using a simpler medium (DMEM: Ham’s F12 (1:1) supplemented with FCS (10%), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), insulin (10 µg/ml) and transferrin (10 µg/ml)) with the same isolation procedure. These attempts were unsuccessful on all occasions (5 eyes).

Ultrastructural analysis of cultured choroidal melanocytes revealed bipolar, spindle-shaped cells containing both primary and mature melanosomes (Fig. 3a). Ocular melanoma cells were either spindle-shaped (Figs. 2f and g, showing a dopa stained culture of cells) or epithelioid (Fig. 2h and Fig. 3). Of 23 ocular melanomas cultured in the simple DMEM: Ham’s F12 medium as described above, all initially attached to the plastic but three failed to grow in culture. The remainder continued to proliferate and many continued for up to six passages. Approximately, one quarter of the tumors had a pure spindle-shaped morphology, another quarter showed a mixture of spindle-shaped and epithelioid morphology which became progressively more epithelioid in culture and the remainder of the tumors were entirely epithelioid. Ultrastructurally these malignant melanoma cells contain irregular melanosomes which are, however, more similar in size and distribution to the normal choroidal melanocyte than to those from RPE cells.

RPE cells contained two types of pigment granules: melanin granules, which are either ovoid or spherical, and lipofuscin granules, which are generally spherical (Fig. 3). Both granules are significantly larger than the melanosomes in either the cultured malignant melanoma cells or choroidal melanocytes (Fig. 3). The lipofuscin granules are less osmiophilic than the melanin granules, hence lighter on electron microscopy.

Comparison of Dopa Staining and Tyrosinase Activity of Normal Choroidal Melanocytes, Malignant Melanoma Cells, RPE Cells, and Skin Melanocytes

We compared the melanogenic potential of all four cell types using dopa staining and by quantifying tyrosi-
sinase enzyme activity (Table 1). Skin melanocytes gave a rapid, strong response to the dopa reaction. Uveal melanocytes also stained with L-dopa although the reaction was not as strong and during a 5- to 12-hour incubation period. Of the five melanoma tumors examined, all eventually were dopa positive on staining, but the reaction took at least 18 hours for four of the tumors examined (see Fig. 2g for example of spindle-shaped melanoma cells stained with L-dopa). RPE cells that had been in culture for several weeks showed no dopa staining, even after an 18-hour incubation. The non-metabolizable substrates, D-dopa and L-dopa, plus the tyrosinase inhibitor phenylthiourea were also included as negative controls for the dopa reaction, and cells staining positive with L-dopa showed barely detectable activity in the presence of the D-enantiomer or L-dopa plus phenylthiourea (results are summarized in Table 1).

We found that the measured tyrosinase activity for all cells was proportional to dopa staining (Fig. 4). Tyrosinase activity in ocular melanocytes was of the same order as that seen in skin melanocytes and murine B16 melanoma cells. Ocular melanoma cells had low levels of tyrosinase activity, as had a freshly isolated preparation of inner choroid cells rich in RPE cells with relatively few melanocytes present (Fig. 4). The latter suggests that even freshly isolated RPE cells contribute little significant tyrosinase activity.

We investigated the effects of culturing the cells with MSH (10^-8 M) for 2 to 6 days. Skin melanocytes did not alter their tyrosinase activity in response to MSH, but ocular melanocytes showed a slight but significant increase in tyrosinase activity as is illustrated in Figure 4 for two separate cultures of choroidal melanocytes. B16 murine melanoma cells, included as positive controls, had a 3-fold increase in tyrosinase activity in response to MSH. Figure 4 also shows that tyrosinase activity in the ocular melanoma cells and a choroidal preparation rich in RPE cells (BM, Fig. 4) failed to respond to MSH with any significant increase in tyrosinase activity.

**Effects of MSH on Melanosomal Size**

We used electron microscopy to estimate the melanosomal size in skin melanocytes, choroidal melanocytes, B16 melanoma, human ocular melanoma, and human retinal pigment epithelial cells. Electron microscopy of human skin melanocytes and human choroidal melanocytes is shown in Figures 5 and 6. Figure 7 summarizes the melanosomal size in the presence and absence of MSH for all cultured cells. In all cases, cells were cultured for 3 days in their appropriate medium with 10^-8 M MSH. For skin melanocytes, MSH produced a slight increase in the size and density of the melanosomes, as can be seen in Figures 5 and 7. Although this increase was statistically significant, the increment in melanosomal size was of the order of 13% for one culture and 5% for another (Fig. 7). In choroidal melanocytes, MSH altered the distribution of melanosomes:

![FIGURE 4. The effect of melanocyte stimulating hormone on tyrosinase activity for murine melanoma cells (B16), skin melanocytes (SM), choroidal melanocytes (CM), inner choroidal preparation (BM), and uveal malignant melanoma cells (MM). Cells were cultured, for 3 days, with (MSH) and without (c) melanocyte stimulating hormone (10^-8 M).](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933180/)
Effect of MSH

FIGURE 5. Electron micrographs of melanocytic dendrites of skin melanocytes, cultured with (MSH) and without (C) melanocyte stimulating hormone. There is a slight increase in size and density of melanosomes in the MSH-treated cells (space bar represents 0.25 μm).

FIGURE 6. Electron micrographs of melanocytic dendrites of choroidal melanocytes, with (MSH) and without (C) melanocyte stimulating hormone. MSH increases the size of melanosomes, and, at lower magnification (a and b), these larger melanosomes are seen to be more equally distributed throughout the melanocyte cytoplasm (space bar represents 1 μm).
cytes to begin comparing the characteristics of these
discussible methodology for culturing $10^7$ cells per eye
with that of the other pigmented cell in the eye, the
cells with that of their transformed counterparts and
methodology for culture of normal choroidal melano-
confirms the conclusions on growth factor require-
culture of uveal melanocytes. Our study essentially
over a period of 2 to 3 months and have compared the
their transformed counterparts. We report a repro-
ments for the uveal melanocyte reached by these au-
 demonstrate that cutaneous melanocytes require a com-
plex growth medium with a wide range of mitogens, as
will be discussed shortly. In this study, we have demon-
strated that a more complex culture medium is re-
quired to culture choroidal melanocytes than that re-
quired to culture ocular malignant melanoma cells,
the majority of which grew successfully in a relatively
simple medium. This clearly implies that they are bio-
logically fundamentally different, in that the growth of
uveal melanoma cells is independent of the hormones
and/or other growth factors essential for the growth of
nonmalignant cells.

Based on the current study, choroidal and skin
melanocytes appear to have similarities in morphology
in culture and in growth medium requirements. An
extensive investigation of the growth requirements of
uveal melanocytes has recently been published\textsuperscript{11} that
essentially confirms that they have similar mechanisms
of control of proliferation to skin melanocytes requir-
ing a cyclic AMP stimulator, bFGF or TPA, and FBS.
The melanocyte medium we used in this study contains
three major mitogenic drives; the phorbol ester PMA,
which is thought to activate and possibly downregulate
protein kinase C; cholera toxin, which is known to ele-
vate cAMP\textsuperscript{12}; bovine hypothalamic extract, which con-
tains bFGF, a known melanocytic mitogen\textsuperscript{13,17,24,25,28}
acting via a tyrosine kinase. Bovine hypothalamic
extract also appears to contain other unidentified brain-
derived mitogens that may stimulate melanocyte
growth.\textsuperscript{24,25} In the recent study of Hu et al,\textsuperscript{11} bFGF
was found to be able to substitute for TPA in culture of
ocular melanocytes confirming the observation ini-
tially made for skin melanocytes by Halaban et al.\textsuperscript{24}

There are, however, some differences between
choroidal and skin melanocytes in culture. In this
study, we found uveal melanocytes will grow to high
density, whereas skin melanocytes, irrespective of
whether they are derived from adult skin or neonatal
foreskin, which are generally more proliferative and
will achieve a greater density in culture, rarely achieve
full cell-to-cell contact even at their highest density in
culture under the conditions described in this report.

Some uveal melanoma cells in culture are morpho-
logically similar to choroidal melanocytes (as can be
seen in Fig. 2g, where spindle-shaped ocular mela-
noma cells are stained for dopa). Melanoma cell mor-
phology is noticeably variable between patients, unlike
normal melanocytes. Uveal melanomas can have a
spindle-shaped, epithelioid, or mixed cellular mor-
phology.\textsuperscript{30,31} Although the spindle-shaped melanoma
cell is similar in morphology to the normal choroidal
melanocyte, epithelioid cells bear no such resem-
blance to their normal precursor cell except that the
melanosome size is equivalent. It is known that the
epithelioid melanomas have a poorer prognosis than
spindle-cell neoplasms.\textsuperscript{30} In culture, we have found
that the growth rate and life span of the melanoma cell
is unpredictable. Although the majority of tumors (20

FIGURE 7. The effect of melanocyte stimulating hormone
($10^{-8}$ M) on melanosome size for murine melanoma cells
(B16), skin melanocytes (SM), choroidal melanocytes (CM),
retinal pigment epithelium (RPE), and uveal malignant mela-
noma cells (MM). The bars represent the mean values ±
SEM of three determinations. MSH increased choroidal me-
lanocyte melanosome size but had no effect on either uveal
melanoma cells or retinal pigment epithelial cells. Statistical
significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

In controls, the largest melanosomes were located pre-
dominantly around the periphery of the cell, whereas
in MSH-treated cells the large melanosomes were dis-
tributed more evenly throughout the cell cytoplasm
(Fig. 6). The increase in melanosome size to MSH was
of the order of 45% and 31% for the two cultures of
melanocytes studied (Fig. 7). Ocular melanoma cells
and RPE cells did not respond to MSH with any
change in melanosome size (Fig. 7).

DISCUSSION

In this study, we aimed to establish a reproducible
methodology for culture of normal choroidal melan-
cytes to begin comparing the characteristics of these
cells with that of their transformed counterparts and
with that of the other pigmented cell in the eye, the
retinal epithelial cell. During the course of this work,
two authoritative studies have appeared from the labo-
ratories of Hu et al\textsuperscript{10,11} establishing conditions for the
culture of uveal melanocytes. Our study essentially
confirms the conclusions on growth factor require-
ments for the uveal melanocyte reached by these au-
thors, that is, that uveal melanocytes, like skin melan-
cytes, require a complex growth medium in contrast to
their transformed counterparts. We report a repro-
ducible methodology for culturing $10^7$ cells per eye
over a period of 2 to 3 months and have compared the
morphology and pigmentary behavior of the melanocy-
to the other pigmented cells in the eye.

Halaban,\textsuperscript{13,24–27} Eisinger,\textsuperscript{12} and Gilchrest\textsuperscript{28} all
demonstrate that cutaneous melanocytes require a com-
Effect of MSH

835

out of 23) became established in culture and began to proliferate, some tumors divided rapidly and continued to do so for many passages, whereas others appeared to cease division after a few passages. The majority of tumors, however, achieved six passages in culture.

Retinal pigment epithelial cells, derived embryologically from the neuroectoderm of the primitive forebrain, are markedly different morphologically to choroidal melanocytes derived from the neural crest. In vivo and in vitro RPE cells contain two types of pigment granules, and their melanosomes are much larger than those of choroidal melanocytes or uveal malignant melanoma cells.

We found that in culture, RPE cells become less differentiated and they fail to survive more than transiently in melanocyte media. Thus, RPE cells can be distinguished from choroidal melanocytes in culture on morphologic grounds and on the basis of their growth requirements.

With regard to the pigmentary behavior of the cells in culture, it is known that skin melanocytes in the lower epidermis produce, and transport, melanin to epithelial keratinocytes. In the eye, we have demonstrated that the melanosomes of the choroidal melanocyte are present at several stages of maturity; they are dopa positive and have appreciable levels of tyrosinase activity similar to that found in skin melanocytes or in murine B16 melanoma cells. In contrast, however, retinal pigment epithelial cells have two types of mature organelle-containing melanin and lipofuscin, respectively.

It is known that RPE cells express mRNA for tyrosinase and have the capacity to produce melanin during embryogenesis, but melanogenesis ceases shortly after birth. It has been reported recently, however, that if grown on an extracellular matrix secreted by bovine corneal endothelial cells, human RPE cells retain pigment at confluence and retain pigment for the long term. Addition of bFGF enhances this effect. Under our culture conditions, however, RPE cells lose their pigmentation and have no detectable tyrosinase activity. Also, in freshly isolated cultures of cells extracted from inner choroidal preparation (which consist largely of RPE cells with fewer than 5% melanocytes), tyrosinase activity is barely detectable, which suggests that enzyme activity is associated with the few choroidal melanocytes present in this rather than RPE cells.

The presence of two totally separate populations of pigment-producing cells in one organ is unique to the eye, and the functions of these pigment cells is not clear. Pigment cells within the eye are probably necessary to prevent internal reflection of light from the sclera back through the retina, and they also prevent incident light from entering through the back of the eye producing conflicting images—a particular problem for some lower vertebrates whose eyes are not embedded in the skull. Recently, it has been suggested that choroidal melanocytes may surround blood vessels and act as filters removing toxic substances and xenobiotic agents from the eye. This theory suggests that melanin may protect the pigmented cells by binding potentially harmful substances, which are slowly released in nontoxic concentrations.

The pigmentation of melanomas is variable but in culture cells from our five tumors had much lower levels of tyrosinase activity than choroidal melanocytes. This is consistent with the relatively low density of melanosomes seen in these cells by electron microscopy. It is unlikely that the differences in tyrosinase activity relate to the rate of proliferation of the cells in culture because some melanocytes proliferated more rapidly than their corresponding tumor cells in vitro and yet had higher levels of tyrosinase activity. It suggests rather that there is an inherent difference in tyrosinase activity in the tumors, which would be consistent with the tumors showing an undifferentiated or poorly differentiated form of normal melanocyte morphology.

In this study, we have undertaken a comparative investigation of the pigmentary behavior of normal choroidal melanocytes, their transformed counterparts, and the retinal pigment epithelial cell. We demonstrate for the first time that choroidal melanocytes respond to MSH by increasing pigmentation as a result of increasing tyrosinase activity and melanosome size. Uveal melanoma cells and retinal epithelial cells do not respond to MSH with any changes in tyrosinase activity or melanosome size under the culture conditions described.

Although skin melanocytes possess MSH receptors capable of binding MSH and show morphologic changes in response to MSH and increases in cyclic AMP to MSH, research in this area has so far failed to demonstrate any significant effect of MSH on melanogenesis in vitro. In this study, we did show a small but significant increase in melanosome size but no appreciable increase in tyrosinase activity for MSH. Previous work from this laboratory has shown that in addition to activating cyclic AMP, MSH is capable of activating protein kinase C in B16 melanoma cells. Information from the recent cloning of the MSH receptor implies that there is a subfamily of receptors coupled to guanine nucleotide-binding proteins. This raises the possibility that in some cells, these receptors may link to signalling pathways other than cyclic AMP and may indeed be involved in biologic actions of MSH other than its effect on pigmentation.

Our finding that choroidal melanocytes respond to MSH in culture with increased tyrosinase activity in contrast to the skin melanocytes requires further investigation.

In summary, we conclude that culture of both ma-
lignant melanoma cells and normal choroidal melano-
cytes from the same eye of a patient is possible. Under
cell culture conditions, both cell types are removed
from their in vivo environment (with its unique endo-
crine and paracrine growth conditions), and are grown
using different culture media. Despite this, we can
conclude that, analogous to the situation that occurs
in the transformation of the skin melanocyte, the ma-
lignant melanoma cell appears to be largely indepen-
dent of the in vitro growth requirements necessary for
the successful culture of the normal ocular melanocyte
and that the ocular melanocyte in culture retains the
ability to respond to MSH, which appears to be lost in
the transformed melanoma.

Key Words
melanocyte, melanoma, melanocyte stimulating hormone,
tyrosinase, RPE

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