Melanogenesis by Human Uveal Melanocytes In Vitro

Dan-Ning Hu,* Steven A. McCormick,* Seth J. Orlow,† Susana Rosemblat,‡ Alexander Y. Lin,* and Kevin Wo*

Purpose. To study melanogenesis by cultured human uveal melanocytes, and the relationship between melanin production by uveal melanocytes in vitro with the degree of iris pigmentation in vivo.

Methods. Melanin content, melanin production, and tyrosinase activity of cultured uveal melanocytes derived from eyes of various iris color were measured at different stages of cultivation.

Results. Cultured uveal melanocytes maintained a constant level of melanin content, expressed tyrosinase activity, and produced measurable amounts of melanin in vitro. Melanosomes in different stages were seen ultrastructurally. Melanin production correlated directly with the degree of iris pigmentation of the eyes from which the uveal melanocytes were isolated. Tyrosinase activity of cultured uveal melanocytes from black versus white donors was significantly different, but, among white donors, there was no correlation with iris pigmentation or with melanin production in vitro.


During the past decade, there has been an increasing interest in the relationship between exposure to visible and ultraviolet light and the development of certain eye diseases, including age-related macular degeneration, senile cataract, and uveal melanoma. Ocular pigmentation, particularly uveal pigmentation, may play an important protective role in prevention of these diseases.11-15 Recently, it has been found that melanin from the uvea could stimulate experimental autoimmune uveitis and may be involved in the pathogenesis of sympathetic ophthalmia and the Vogt-Koyanagi-Harada syndrome.11-13

Although many studies of melanogenesis by epidermal melanocytes have been reported and it is well established that epidermal melanocytes do produce melanin in vivo and in vitro,14-26 little is known about melanogenesis by uveal melanocytes. Whether uveal melanocytes produce melanin in vivo during adulthood remains controversial.27-32 Little work has been performed on melanogenesis by uveal melanocytes in vitro.33,34 Investigation of melanogenesis by uveal melanocytes in vitro has been hampered by an inability to obtain a sufficient number of pure uveal melanocytes for study.

We have developed methods for isolation and culture of human uveal melanocytes. This culture system is capable of generating large numbers of human uveal melanocytes in pure culture. We have now established many cell strains from the iris, ciliary body, and choroid from donors of different races with various degrees of iris stromal pigmentation (iris color). The purpose of the present study was to investigate melanogenesis by uveal melanocytes in vitro. We wanted to explore specifically whether uveal melanocytes produce melanin in vitro, whether melanin content and melanin production by uveal melanocytes in vitro correlate with iris pigmentation, whether cultured uveal melanocytes express tyrosinase activity, and whether tyrosinase activity of uveal melanocytes...
correlates with iris pigmentation, melanin content and melanin production. We also wanted to study factors influencing melanin content and production, as well as the tyrosinase activity of uveal melanocytes in vitro.

METHODS

Cell Culture

Uveal melanocytes were isolated and cultured from adult donor eyes as described previously. Briefly, a circumferential scleral incision was made at the ora serrata, separating the globe into anterior and posterior portions. The iris was excised and placed in a dish with the posterior surface upward. The iris pigment epithelium was separated from the stroma after immersion in 0.25% trypsin solution (Gibco, Grand Island, NY) at 37°C for 1 to 2 hours. The remaining iris stroma was placed in 0.25% trypsin solution at 4°C for 18 hours, followed by incubation at 37°C for 1 hour. The isolated cells were collected. The trypsin solution was replaced by collagenase solution (400 U/ml, in F-12 medium, Sigma, St. Louis, MO) and incubated at 37°C. The collagenase solution was replaced, and the cells were collected, centrifuged, resuspended, and plated each hour for 3 hours. The ciliary body was separated from the sclera and placed in a culture dish with its inner surface upward. The ciliary epithelium was separated from the ciliary body after immersion in 0.25% trypsin solution (Gibco) at 4°C for 30 minutes, rinsed with distilled water, incubated in 0.25% collagenase solution (Sigma) at 37°C for 2 to 3 hours. The isolated uveal melanocytes were cultured in Falcon culture dishes (Becton Dickinson, Oxnard, CA) with F12 medium, which consisted of F-12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine (all from Gibco), 10 ng/ml cholera toxin, 50 μg/ml gentamicin (all from Sigma), and 20 mg/ml basic fibroblast growth factor (Promega, Madison, WI). The culture dishes were incubated in a humidified 5% CO2 atmosphere. The medium was changed three times a week. Gentamicin (Sigma), a cytotoxic agent, was added (100 μg/ml) for 3 to 7 days when necessary to eliminate contaminating cells. Fibroblasts and pigment epithelial cells are much more sensitive to gentamicin than are uveal melanocytes. At confluence, the uveal melanocytes were detached by trypsin-EDTA solution, diluted 1:3 to 1:4, and subcultured.

The 10 cell strains of uveal melanocytes used in the present study were isolated from donors with different iris color. Iris pigmentation was classified into three categories: light pigmentation (1+), blue to light yellow-green, four cell strains (two from the iris and two from the choroid); moderate pigmentation (2+), green or brown, five cell strains (three from the iris, one from the ciliary body, and one from the choroid); dense pigmentation (3+), dark brown, one cell strain from the iris of a black donor. The research was conducted in accordance with the tenets of the Declaration of Helsinki, and was approval was granted by institutional human experimentation committee.

Melanin Measurement

Cultured uveal melanocytes were detached by trypsin-EDTA solution and counted in a hemocytometer, the cell suspensions were centrifuged, and the pellet was dissolved in 1 N NaOH. Melanin concentration was determined by measurement of optic density at 475 nm and compared with a standard curve obtained using synthetic melanin (Sigma). Melanin content was expressed as ng/cell.

Calculation of Melanin Production

Melanin production was calculated by determining the melanin content and the cell counts at the beginning and the end of each generation by the following formula:

\[ C_p = C_o P - C_o / 1.3D(P - 1) \]

where \( C_o \) and \( C_i \) represent the melanin content per cell at times 0 and time \( t \), respectively; \( P \) is the population increase during time \( t \), \( D \) is the doubling time of the uveal melanocytes; and \( C_o \) is melanin production per cell per day during time \( t \).

DOPA Reaction

Uveal melanocytes cultured in chamber slides were fixed with 5% formalin in phosphate buffer (pH 7.0) at 4°C for 30 minutes, rinsed with distilled water, incubated with 0.1% L-DOPA (3,4-dihydroxyphenylalanine, Sigma) in phosphate buffer at 37°C for 3.5 hours with one change of solution, then fixed with 10% formalin in phosphate buffer at 25°C for 1 hour, air-dried, coverslipped, and examined by light microscopy. Two cell strains of retinal pigment epithelial cells isolated from adult human eyes (5th and 10th generation) and two cell strains of fibroblasts isolated from human sclera (5th generation and 7th generation) were tested as controls (both cell types were cultured with F12 medium supplemented with 10% fetal bovine serum).

Tyrosinase Activity

Tyrosinase activity was evaluated in nine cell strains using an adaptation of the Pomerantz method. Tyrosinase activity was expressed in terms of melanin production per cell per day during time \( t \).

\[ C_p = C_o P - C_o / 1.3D(P - 1) \]
Melanogenesis by Human Uveal Melanocytes

1.0 : 0.1 : 0.01 : Melani Com snt (ng/cell)

**FIGURE 1.** Melanin content (ng/cell) of two cell strains of cultured human uveal melanocytes (UM) at various stages during cultivation. Uveal melanocytes from light green iris (solid line) and from brown iris (dashed line), which is based on the measurement of $^3$H$_2$O released by the enzymatic hydroxylation of tyrosine.

**Ultrastructure**

Cultures of uveal melanocytes in various stages were fixed, embedded in Epon, stained with lead citrate–uranyl acetate, and examined by routine transmission electron microscopy.

**RESULTS**

Pure cultures of human uveal melanocytes were established from the iris, ciliary body, and choroid using the described methods. Most of the uveal melanocytes attached and spread within 1 to 3 days after plating. After 6 to 12 days, most of the spread cells had divided. The dividing cells showed a gradual dilution of pigment. Each cell developed two or more dendritic processes. The uveal melanocytes grew quickly upon subculture, and the pigment content became stable during the active growth stage. These cell strains have been passaged for 15 to 30 generations over 3 to 7 months, with 30 to 45 divisions. The doubling time was 2 to 3 days during the active growth stage. When the cultures became senescent, the cytoplasm of the uveal melanocytes spread to form round or polygonal platelike configurations, and the pigment content gradually increased.

**Melanin Contents**

Melanin content per cell in cultured uveal melanocytes decreased rapidly during early passages and then stabilized. Despite the dilutional effect of cell division, the melanin content per cell remained stable in each cell strain during the active growth stage. The melanin content increased after the cells became senescent (Fig. 1).

Melanin content of cultured uveal melanocytes during active growth stage varied from 0.0118 ng/cell to 0.102 ng/cell in the 10 cell strains tested. The melanin content increased after the cells became senescent (Table 1). The difference of melanin content between actively growing cells and senescent cells was statistically significant ($P < 0.01$).

The melanin content of iridal melanocytes from a black donor during active growth stage and senescent stage were significantly higher than those from white donors ($P < 0.01$).

During the active growth stage and the senescent stage, the melanin content of uveal melanocytes from moderately pigmented eyes was significantly greater than that from lightly pigmented eyes but less than that from the darkly pigmented eye (Table 1). Correlation tests showed that the melanin content of uveal melanocytes in the active growth stage or senescent stage correlated well with iris color ($P < 0.01$).

Melanin content of uveal melanocytes isolated from the iris, ciliary body, and choroid did not show significant differences.

The melanin content of uveal melanocytes at different stages of culture correlated with the growth rate within a particular cell strain. Correlation tests showed that melanin content correlated well with the doubling time in four cell strains in which the doubling time and melanin content were measured in each generation from primary culture to senescence ($P < 0.01$).

**Melanin Production**

Melanin production was $0.0070 \pm 0.0043$ ng/cell per day (mean ± SD) in the 10 cell strains tested during the active growth stage, continued to increase in early senescence ($0.0181 \pm 0.0130$ ng/cell per day), and decreased to a slightly lower level ($0.0151 \pm 0.0124$ ng/cell per day) in senescent cells that remained stationary for a long period (Fig. 2).

Melanin production by iridal melanocytes from a black donor during active growth and senescent stages were also significantly higher than those from white donors ($P < 0.01$).

Melanin production by uveal melanocytes from moderately pigmented eyes was significantly greater than that from lightly pigmented eyes and less than that from the darkly pigmented eye (Table 1). Correlation tests showed that melanin production of uveal melanocytes in either the active growth or senescent stage correlated well with iris color ($P < 0.01$).

Melanin production also correlated with growth rate in four cell strains with complete data ($P < 0.01$).

**DOPA Test**

The DOPA tests in the four cell strains of cultured uveal melanocytes tested revealed positive results (Fig.
TABLE 1. Melanin Content, Melanin Production, and Tyrosinase Activity of Cultured Melanocytes From Eyes of Various Iris Color

<table>
<thead>
<tr>
<th>Iris Color</th>
<th>Melanin Content (ng/cell)</th>
<th>Melanin Production (ng/cell per day)</th>
<th>Tyrosinase Activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth Stage</td>
<td>Senescent Stage</td>
<td>Growth Stage</td>
</tr>
<tr>
<td>Light</td>
<td>0.0140</td>
<td>0.0978</td>
<td>0.0041</td>
</tr>
<tr>
<td></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>Moderate</td>
<td>0.0249</td>
<td>0.2777</td>
<td>0.0071</td>
</tr>
<tr>
<td></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
<tr>
<td>Dark</td>
<td>0.1020</td>
<td>0.6980</td>
<td>0.0180</td>
</tr>
</tbody>
</table>

5), whereas all cultured retinal pigment cells and fibroblasts were negative.

**Tyrosinase Activity**

The tyrosinase activity varied from 19.2 U to 115.4 U (a unit of tyrosinase was defined as the activity of enzyme that catalyzed the hydroxylation of 1 pmol of tyrosine/mg protein per hour) in the eight cell strains tested during the active growth stage, and it increased after senescence (Table 1).

The tyrosinase activity of iridal melanocytes from a black donor during the active growth stage and the senescent stage were also significantly higher than those from white donors (*P < 0.01*).

However, in the uveal melanocytes from white donors, tyrosinase activity of uveal melanocytes from moderately pigmented eyes did not differ significantly from lightly pigmented eyes in either the active growth stage or the senescent stage (Table 1). The correlation coefficients between iris pigmentation and tyrosinase activity of uveal melanocytes from white donors were −0.4209 (*P > 0.05*) during active growth stage and −0.3193 (*P > 0.05*) in senescence.

The tyrosinase activity of uveal melanocytes correlated with melanin content and melanin production in uveal melanocytes in the active growth stage (*P < 0.05*) but did not correlate with cultures in the senescent stage (*P > 0.05*). After excluding the uveal melanocytes from the black donor, the tyrosinase activity in the uveal melanocytes from white donors did not correlate with melanin content or melanin production in either the active growth stage or in the senescent stage (*P > 0.05* in all correlation coefficients).

**FIGURE 2.** Melanin production (ng/cell per day) of two cell strains of uveal melanocytes at various stages during cultivation. Uveal melanocytes from light green iris (solid line) and from brown iris (dashed line).

**FIGURE 3.** DOPA test of cultured human uveal melanocytes. (A) Before DOPA test. Magnification, ×250. (B) DOPA test revealed positive reaction. Magnification, ×200.
Ultrastructural Study

Transmission electron microscopy revealed premelanosomes and all stages of melanosomes (stages I to IV) in the cytoplasm of cultured uveal melanocytes (Fig. 4). In actively growing cells, there were fewer melanosomes, and most were mature melanosomes (stage IV). In early senescent stage cells, many premelanosomes and immature melanosomes (stages I to III) appeared in the cytoplasm, indicating active melanosome biogenesis and melanogenesis. In late senescence cells, mature melanosomes again predominated.

The size of melanosomes in uveal melanocytes from the black donor (0.35 μm X 1.02 μm) was significantly larger than those from white donors (0.25 μm X 0.71 μm) (P < 0.01).

DISCUSSION

In the past decade, extensive studies of melanogenesis by epidermal melanocytes have been presented. 14-26 Not only do epidermal melanocytes produce melanin and transfer it to keratinocytes in vivo, they also express tyrosinase activity and synthesize melanin in vitro. 14-25

Controversy exists concerning the capacity of uveal melanocytes for melanogenesis in vivo in adulthood. The traditional view is that the uveal melanocytes produce melanin before and after birth, leading to increased pigmentation of the iris within the first several months of life. It was considered that melanogenesis then ceased and that all melanosomes in the uveal melanocytes were fully mature by 10 months. 30 However, stage III melanosomes have been found in iridal melanocytes in the adult monkey. 27 Some authors reported that tyrosinase activity could be demonstrated in adult human uvea31 and mature rabbit iris and choroid. 32 However, others have reported that tyrosinase and related proteins were synthesized only early after birth. 25 These various reports leave unresolved the question whether the capacity for melanin production is retained by the uveal melanocytes in vivo in the adult.

Other than a few reports of positive DOPA reactions and measurable tyrosinase activity in cultured choroidal melanocytes, little is known about melanogenesis by uveal melanocytes in vitro. 33-36 For accurate investigation of melanogenesis in vitro, it is essential to generate a large population of the iris within the first few generations of cultured uveal melanocytes before isolation and cultivation of pure cultures of human uveal melanocytes. 35 A culture system of human uveal melanocytes has been developed that can provide large numbers of pure human uveal melanocyte cells. 35,36 Therefore, measurement of melanin content, melanin production, and tyrosinase activity of uveal melanocytes in vitro became possible.

The present study revealed that cultured human uveal melanocytes isolated from iris, ciliary body, or choroid maintained a constant level of melanin content even during the active growth stage. They also produced measurable amounts of melanin in vitro and showed a positive DOPA reaction. Appreciable levels of tyrosinase activity also were demonstrated. Melanosomes in different stages were seen ultrastructurally. These results indicate that uveal melanocytes, similar to epidermal melanocytes but in contrast to retinal pigment epithelium, can produce melanin in vitro.

Melanin content and the production of melanin by uveal melanocytes in vitro apparently are influenced by racial and genetic factors because uveal melanocytes from a black donor showed significantly higher melanin content, rate of melanin production, and tyrosinase activity, as well as larger melanosomal size, than seen in uveal melanocytes derived from white donors.

In white donors, melanin content and melanin production in cultured uveal melanocytes correlated well with iris pigmentation in vivo, indicating that cultured uveal melanocytes retain their different capacities for melanin production ability in vivo well. Therefore, cultured uveal melanocytes provide an excellent model system for studying melanogenesis in uveal melanocytes.

The content of melanin per cell in cultured uveal melanocytes is not only determined by the production of melanin but also by growth rate. In stationary cells, the melanin produced accumulates within the cell and results in a rapid increase of melanin content per cell. In growing cells, the melanin is diluted to daughter cells during division. If the melanin production rate equals the rate of dilution, the melanin content per cell would remain unchanged. In rapidly growing cells, if the dilution rate is greater than that of melanin production, melanin content per cell would decrease, as we observed in the first few generations of cultured uveal melanocytes.

Melanin production by cultured uveal melanocytes also correlated with the growth rate in four cell strains when compared to the doubling time in each generation. Presumably, stationary cells arrested in the G1 stage of the cell cycle have a greater proportion of cellular metabolism to the production of melanin than do rapidly growing cells. Melanin production of uveal melanocytes decreased to a lower level in senescent cells that remained stationary for long periods. This finding is
FIGURE 4. Transmission electron microscopy of cultured uveal melanocytes (UM) demonstrating premelanosomes and melanosomes in various stages of maturation (stages I to IV). (A) Uveal melanocytes from a black donor during growth stage showing mitochondria (M), endoplasmic reticulum (E), nuclei (N), and melanosomes. Most melanosomes are stage IV, whereas only a few are stage II or stage III melanosomes. Magnification, ×6,000. (B) Uveal melanocytes from a white donor during the early senescence stage showing numerous melanosomes, most of which are stage I and stage II melanosomes. Relatively few are stage III melanosomes. Magnification, ×15,000. (C) Uveal melanocytes from a white donor during the late senescence stage demonstrated an increased number of stage III and stage IV melanosomes (compare to Fig. 5B). Some stage II melanosomes also are present. Magnification, ×20,000.

consistent with results indicating that tyrosinase activity of cultured epidermal melanocytes was inhibited by high concentrations of melanin and that virtually no tyrosinase activity could be identified in fully mature melanosomes (stage IV).  

Tyrosinase is thought to control the rate limiting step on the enzymatic production of melanin in epidermal melanocytes. Therefore, tyrosinase activity is thought to be the major regulatory factor in melanogenesis. In the present study, the relationship between tyrosinase activity of uveal melanocytes in vitro with iris pigmentation in vivo seems more complicated. Uveal melanocytes from the eye of a black donor contained higher tyrosinase activity than eyes from white donors. This is consistent with previous reports that epidermal melanocytes derived from black skin expressed higher tyrosinase activity than those from white skin. However, when the tyrosinase activity of cultured uveal melanocytes from eyes with light irides was compared to that from dark irides, approximately equivalent levels of tyrosinase activity was present in the two groups. Moreover, in uveal melanocytes from white donors, tyrosinase activity did not correlate with melanin content and melanin production in vitro, indicating that, although tyrosinase activity attributed to racial difference was present in cultured uveal melanocytes; within the same race, tyrosinase activity in vitro did not correlate with iris pigmentation in vivo. This may be explained by the complexity of the role of tyrosinase in melanogenesis.

Tyrosinase holds a central position in the biosynthesis of melanin because of its ability to catalyze the first two rate-limiting reactions, namely the hydroxylation of tyrosine to DOPA and its subsequent oxidation to dopaquinone. It was previously thought that the subsequent steps proceeded more or less spontaneously, ending with the format of melanin. However, more recently, it has been found that many other factors regulate melanogenesis, such as the activity of auxiliary enzymes (e.g., dopachrome tautomerase and peroxidase) and certain metal ions, especially copper and iron. An additional level of melanogenic control exists even before tyrosinase. Therefore, tyrosinase activity is important, but it is not the sole factor for determining the rate of melanin production. Many other factors may modify the biosynthesis of melanin. These concepts may explain our findings that different levels of melanin production existed in uveal mela-
Melanogenesis by Human Uveal Melanocytes

...tyrosinase activities.

Regulation of melanogenesis in uveal melanocytes may have significance in the pathogenesis of several eye diseases. Recently, it was found that development of certain eye diseases may be related to exposure to visible and ultraviolet light. Uveal pigmentation could protect intraocular tissues from irradiation and may play a role in the prevention of these disorders. For example, a population-based study indicated that exposure to sunlight may be associated with age-related macular degeneration. Near-ultraviolet radiation is thought to be one of the factors responsible for oxidative changes in lens protein in senile cataract. Epidemiologic studies have shown a high correlation between increased ultraviolet-B exposure and human cortical cataract formation. Sunlight exposure has been identified as a possible risk factor for the development of intraocular melanoma. Persons with blue irides have a significantly greater risk for ocular melanoma than those with brown irides. 

Recent studies demonstrated that purified uveal melanin was uveitogenic and could induce severe experimental autoimmune anterior uveitis. Ocular immune response to modified melanocytic autoantigens may play a role in the pathogenesis of sympathetic ophthalmia and Vogt-Koyanagi-Harada syndrome. Although uveal pigmentation may play an important role in the physiology and pathology of the eye, little is known about melanogenesis by uveal melanocytes in vivo or in vitro. We have developed a method for uveal melanocytes culture, and we have demonstrated that cultured uveal melanocytes can synthesize melanin in vitro and that melanin content and melanin production of uveal melanocytes in vitro correlate with iris pigmentation in vivo. It is now possible to use this model system to study melanogenesis by uveal melanocytes and to explore the functions of melanin in the eye and the role of melanin in the pathogenesis of various eye diseases.

Key Words
melanogenesis, uveal melanocytes, melanin production, tyrosinase, iris pigmentation

References