Expression of melanin-related genes in cultured adult human retinal pigment epithelium and uveal melanoma cells

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Purpose: Controversy exists over melanogenesis of adult human RPE cells in vitro. This study investigated melanin content and production and expression of tyrosinase (TYR), tyrosinase-related-protein-1 (TRP1), tyrosinase-related-protein-2 (TRP2), and P gene in cultured human RPE cells and uveal melanoma cells.

Methods: RPE cells were isolated and cultured from three adult donor eyes. A continuous human uveal melanoma cell line was established from primary choroidal melanoma. Melanin content and production were measured, and the expression of TYR, TRP1, TRP2, and P gene at the mRNA and protein levels were detected by RT-PCR and western blot, respectively.

Results: Melanin content per cell of cultured RPE decreased rapidly and in proportion to cell division. No melanin production could be demonstrated in any passages. In cultured RPE cells, mRNA expression of TYR, TRP1, TRP2, and P gene and protein expression of TYR, TRP1, and TRP2 could not be detected. In uveal melanoma cells, melanin content per cell remained stable, and melanin production could be detected in each passage. Expression of mRNA of TYR, TRP1, TRP2, and P gene and protein of TYR, TRP1, and TRP2 could be detected in melanoma cells.

Conclusions: Human RPE cells under standard culture circumstances do not produce melanin and do not express the four key genes required in melanin biosynthesis pathway. In contrast, human uveal melanoma cells produce melanin and express all of these melanogenic genes in vitro.

Melanin can be found in various types of pigment cells in the skin, eye, and other tissues. In the eye, there are two different types of pigment cells: the pigment epithelium originated from neural ectoderm and uveal melanocytes from neural crest. Both pigment epithelium and uveal melanocytes produce and store melanin in the melanosomes.

Young, unbleached melanin is an antioxidant, a free radical scavenger and has an affinity for various chemical substances [1-4]. Young melanin in the retinal pigment epithelium (RPE) filters toxic substances from the choroid and protects the retina from oxidative and chemical stress [5-7]. Aging processes in the RPE may diminish the protective properties of melanin [8,9]. In turn, this may lead to damage of the retina, initiating various ocular diseases, including age-related macular degeneration [10-12].

Studies on epidermal melanocytes found that there are at least two different types of melanin: eumelanin, which is a black or brown color, and pheomelanin, which is a yellow to reddish color. Melanins in the RPE are mainly eumelanin [13]. Enzymes involved in eumelanin biosynthesis include tyrosinase, tyrosinase-related-protein-1 (TRP1) and tyrosinase-related-protein-2 (TRP2) [14]. In addition to the tyrosinase family, pink-eyed dilution locus in mice also plays an important role in melanin biosynthesis. It has been reported that most eye color variations in Europeans are due to polymorphism in the P gene, which is the human homolog of pink-eyed dilution locus [15,16].

All of these genes and gene products have been discovered from studies in epidermal melanocytes and cutaneous melanoma cells. Little is known about the expression of these genes in ocular pigment cells [17-20]. Human RPE cells contain a large amount of melanin, which is produced mainly during the prenatal period. There is controversy over the melanogenic activity of adult human RPE cells. Although a number of authors have said adult human RPE cells do not produce melanin in vitro under standard culture circumstances, there has yet to be published quantitative studies on melanin content and production in cultured human RPE cells [2,10,21-23]. In addition, the expression of P gene in cultured RPE cells as well as the expression of tyrosinase, TRP-1, and TRP-2 key genes involved in melanin biosynthesis in cultured human RPE cells has not been studied thoroughly [17-19].

While the expression of the tyrosinase gene family has been systematically studied in cutaneous melanoma cell lines, but not in human uveal melanoma cell lines, a few studies have indicated these cells perhaps likely produce melanin and possess tyrosinase activity in vitro [24-28]. The present study examined the expression of tyrosinase, TRP-1, TRP-2, and P gene in melanin biosynthesis in cultured adult human RPE cells and human uveal melanoma cell lines. RT-PCR and western blotting analysis was combined...
with melanin content measurement and melanin production studies to obtain a more precise clarification of melanogenesis in these two different ocular pigment cells.

**METHODS**

**Tissue source:** This study was approved by the Ethics Committee of Wenzhou Medical College, and it complied with the tenets of the Declaration of Helsinki for Research Involving Human Tissue. Three adult human eyes were obtained from the Wenzhou Eye Bank. All donors age 23-28 were free of ocular diseases. Prior to their death, donors had signed informed consent in regard to the voluntary donation of eyeballs for organ transplantation and medical research.

**Cell culture:** Donor eyeballs were enucleated within 4 h after death and were used immediately for cell culture once corneas were removed for corneal transplantation. RPE were isolated and cultured as follows [2]. A circumferential incision was made in the sclera of the donor eye 8 mm behind the limbus. The posterior segment, including the vitreous, retina, choroid and the posterior sclera was placed in a culture dish. The vitreous and the retina were excised under a dissecting microscope. RPE were immersed in a trypsin (0.05%)-EDTA (0.02%) solution (Sigma, St. Louis, MO) at 37 °C for 1 h. Culture medium with 10% serum was added and the RPE were isolated and collected with a fire-blown Pasteur pipette under direct observation using a dissecting microscope. Isolated cells were centrifuged, re-suspended and seeded to a culture flask. The culture medium used for culturing the RPE was Nutrient Mixture Ham’s F12 medium supplement with 10% fetal bovine serum (FBS) (Invitrogen, Carsbad, CA). Cells were cultured in a humidified, CO2-regulated incubator (95% air/5% CO2) atmosphere. The vitreous and retina were excised under a dissecting microscope. RPE were immersed in a trypsin (0.05%)-EDTA solution (Sigma, St. Louis, MO) at 37 °C for 1 h. Culture medium with 10% serum was added and the RPE were isolated and collected with a fire-blown Pasteur pipette under direct observation using a dissecting microscope. Isolated cells were centrifuged, re-suspended and seeded to a culture flask. The culture medium used for culturing the RPE was Nutrient Mixture Ham’s F12 medium supplement with 10% fetal bovine serum (FBS, 2 mM glutamine and 50 µg/ml gentamicin (Invitrogen, Carsbad, CA). Cells were cultured in a humidified, CO2-regulated incubator (95% air/5% CO2) atmosphere. The culture medium was changed three times a week. Once cells reached confluence, they were detached from the culture dish using the trypsin-EDTA solution. The cell suspension was diluted at 1:3 and seeded into culture flasks for subculture [2]. Cell lines of passages three to five were used in the studies.

Uveal melanoma cell line SP6.5 was supplied by Dr. G. Pelletier (Research Center of Immunology, Quebec, Canada). This cell line was isolated from a choroidal melanoma patient (melanotic melanoma) and was tumorigenic to immune-nude mice [29]. This cell line were cultured for 20 generations in Dulbecco’s Modified Eagle Medium (Invitrogen, Carsbad, CA) with 10% FBS in New York Eye and Ear Infirmary laboratory. This cell line has been cultured in New York Eye and Ear Infirmary laboratory for more than 5 years, having been passed for over 200 generations with more than 300 divisions, indicating this is a continuous cell line.

**Measurement of melanin content and production:** Melanin content per cell and melanin production were measured as follows [20]. Cultured RPE or melanoma cells were detached by trypsin-EDTA solution from the culture flask and counted in a hemocytometer. Cell suspensions were centrifuged, and the pellet was dissolved in 1 N NaOH. Melanin concentration of the NaOH-melanin solution was determined by measurement of optic density at 475 nm by a spectrophotometer and compared with a standard conversion curve (with reading as the vertical axis and melanin content as the horizontal axis) obtained using melanin from septa (Sigma, St. Louis, MO). Melanin content was expressed as ng/cell [20].

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:

\[ C_p = C_0 \cdot P - C_0 / 1.3 \cdot D(P-1) \]

where \( C_0 \) and \( C_p \) 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 UM; and \( C_p \) is melanin production per cell per day during time t [20].

**Reverse transcription polymerase chain reaction:** Total RNA was extracted from both RPE cells (at third to fifth passages) and melanoma cells with Trizol reagent (Invitrogen) and confirmed using spectrophotometry and formaldehyde/agarose gel electrophoresis. Contaminated genomic DNA, was removed by treating total RNA with 2.5 U RNase-free DNase I (Invitrogen) at 37 °C for 30 min and then heating with 4 mM EDTA at 65 °C for 10 min. The reverse transcription step was performed at 42 °C for 60 min in 20 µl solution containing 5 µg DNAse-treated RNA, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 5 mM dithiothreitol, 20 U of RNase inhibitor, 0.5 µg oligo(dT) [12-18], 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, and 200 U RNAse H-free reverse transcriptase (Invitrogen).

Specific primers were designed based on published nucleotide sequences for tyrosinase, TYRP1, TYRP 2, and P gene (Table 1) [30,31]. PCR was performed in a 50 µl solution containing 2 µl of RT reaction products, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 0.2 µM of upstream primer, 0.2 µM of downstream primer, and 2.5 U Taq DNA polymerase. The PCR product were electrophoresed on 1.5% agarose gels, which contained 1 µg/ml ethidium bromide, and then photographed under ultra-violet illumination. Product length was estimated using a standard 250 bp DNA ladder as a size marker. Each PCR product was identified by DNA sequencing.

**Western blot analysis:** The RPE cells at third fifth passages and melanoma cells were harvested under centrifugation. Cells were boiled in a sample buffer that contained 50 mM Tris-HCl, (pH 6.8), 10% glycerol, 2% SDS, 0.1 M
dithiothreitol, 0.001% bromphenol blue, 2 mM EDTA along with a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were quantified with Bio-Rad Protein Assay kit (Bio-Rad Lab., Hercules, CA) using bovine serum albumin as the standard. Next, 30 µg of the lysates were loaded in each lane of 12% SDS-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane (Amersham Life Science, Arlington Heights, IL) for electrophoresis and then blocked overnight in 5% fat-free dry milk, 0.1% Tween 20, 150 mM NaCl, and 50 mM Tris (pH 7.5). Three antibodies, anti-tyrosinase, anti-TYRP1 and anti-TYRP 2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), were used in western blot. The specificity of the antibodies had been confirmed by the manufacturer using preabsorption control. Blots were incubated with primary antibodies diluted at 1:500 in tris-buffered saline with Tween for 2 h at room temperature and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (diluted at 1:3000). The target proteins were detected using enhanced chemiluminescence.

RESULTS

Melanin content and melanin production: Cultured RPE cells grew well with a doubling time of 48-60 h. Melanin content per cell decreased rapidly and in proportion to cell division in the first eight passages (Figure 1). No melanin production could be demonstrated in any passages (from primary culture to eighth subculture).

In contrast to RPE cells, melanin content per cell in uveal melanoma cells remained stable despite the dilutional effect of cell division in the first eight passages (Figure 2). Melanin content of cultured uveal melanoma cells during 15 consecutive passages varied from 0.0173 to 0.0204 ng/cell (0.0186±0.0011 ng/cell, mean±SD).

Melanin production could be detected in cultured melanoma cells in each passage. Melanin production also remained stabilized and was 0.00516±0.00037 ng/cell/day (mean±SD) during 15 consecutive passages (Figure 2).

mRNA expression of tyrosinase, TYRP1, TYRP2, and P gene: Results of the RT-PCR analysis of total RNA isolated from RPE and melanoma cell lines of human eyes are shown in Figure 3. mRNA expression of tyrosinase, TYRP1, TYRP2, and P gene were present in uveal melanoma cells, and the size of the products amplified by the PCR primers matched the predicted size of the individual gene (Figure 3). All PCR products were further confirmed by DNA sequencing. None of these four genes were detected in RPE cells.

Presence of the proteins of tyrosinase, TYRP1, and TYRP2: Western blot analysis indicated clearly that there was no expression of tyrosinase, TYRP1, and TYRP2 in RPE when compared with melanoma cells (Figure 4). These three proteins were expressed strongly in melanoma cells. Western blot of P protein was not performed due to the lack of availability of a P protein antibody.

DISCUSSION

At the time of birth, humans have a substantial amount of melanin in their RPE cells. Melanogenesis occurs in the RPE early in the fetus, with immature melanosomes visible as early as seven weeks of gestation. Between the eighth and 14th week of gestation, melanosomes at all stages of maturation can be observed. Within a few weeks, melanin production ceases. Polymerization of melanin continues to occur in melanosomes until humans reach approximately two years old, when the RPE contains only mature melanosomes [10]. There is controversy over melanogenesis of RPE after birth [32]. Very little or no tyrosinase activity could be detected in adult bovine RPE cells [33,34]. Melanin content of RPE decreases significantly in aged eyes [3-5]. Therefore, melanin biosynthesis is either absent in adult human RPE cells or occurs only at a slow rate.

In the RPE, eumelanin is the main type of melanin [13]. Enzymes and proteins involved in eumelanin biosynthesis include tyrosinase, TRP-1, TRP-2, and P protein.
Tyrosinase locus (TRP) maps to human chromosome 11q14-21. Tyrosinase gene codes tyrosinase, which is a rate limiting enzyme in melanin synthesis. Tyrosinase is able to catalyze the oxidation of tyrosine to dopaquinone directly [35]. Mutation in the TRP gene has been found to be associated with ocucutaneous albinism type 1 [14]. TRP1 maps to human chromosome 9p23 and encodes 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase, which promotes the DHICA oxidization and finally converts to eumelanin in murine but not in human melanocytes. It is thought that the main function of TRP1 in humans is to stabilize and to maintain tyrosinase protein levels [36,37]. Mutation in the TRP1 gene has been associated with ocucutaneous albinism type 3 [14]. TRP2 gene maps to human chromosome 13q32 and codes for an enzyme TRP2 with dopachrome tautomerase activity, which catalyzes the conversion of dopachrome to DHICA. Mutation of TRP2 in mice results in a dilution of the coat color and slightly yellowish ear (slaty mice). At present, there has been no reported mutation of the human TRP2 gene.

The pink-eyed dilution locus and its human homolog, P (maps to human chromosome 15q11.2-q12), encodes a protein of 110 kDa with 12 membrane-spanning domains localized to the melanosome membrane. The P protein is required for normal melanin (primarily eumelanin) biosynthesis. Mutation in the human P gene is associated with ocucutaneous albinism type 2 [14,38,39]. Most normal variations in eye-color in Europeans are due to polymorphism of the P gene [15,16].

Adult human RPE cells do not produce melanin in vitro under ordinary culture circumstances [2,10,17,21-23]. Methodology for the measurement of melanin content and production of ocular melanocytes was established ten years ago [20]. However, measurement of melanin content of RPE cells in consecutive generations and quantitative study of production of melanin by cultured RPE cells have not been reported previously. The expression of the tyrosinase gene family in cultured adult human RPE cells has not been studied thoroughly. Abe et al. reported that tyrosinase and TRP1 mRNA could not be detected in established adult human RPE cells in vitro. However, Abe et al. did not study the protein expression of these two enzymes and the expression of TRP2 [18,19]. Smith-Thomas et al. reported that protein of TRP1 and TRP2 could not be detected in cultured human RPE cells, but they used only the non-quantitative immunostaining method in their studies [17]. The expression of the P gene, which is the main factor determining most human eye-color variation, has never been reported.

It is not sufficient to verify the presence of a specific gene in cells using a single method, because each method has its own limitation. Therefore, this study was designed to detect the presence of a group of key genes related to melanogenesis in cultured human RPE cells at mRNA and protein levels by standard molecular biology methods. Quantitative study of melanin content and production in these cells over many consecutive generations were measured and calculated to reveal melanogenic activity of adult human RPE cells in vitro.

In the present study, melanin content per cell in cultured RPE cells decreased rapidly and in proportion to cell division. No melanin production could be demonstrated in vitro. RT-PCR showed that there was no mRNA expression of tyrosinase, TRP1, TRP2 and P-genes in cultured adult human RPE cells. Western blot analysis demonstrated the absence of the proteins of tyrosinase, TRP1 and TRP2 in these cells. In contrast to the RPE cells, the mRNA and proteins of these genes could be detected in uveal melanoma cells by RT-PCR and western-blot analysis.

Our study showed no melanin biosynthesis activity could be detected in cultured human RPE cells under standard culture circumstances. Therefore, cultured RPE cells are not adequate for studying melanogenesis of RPE or for performing experiments requiring pigmented RPE cells. Several authors reported cultured adult human RPE may produce melanin under special circumstances or induced by certain stimulators [40-45]. Dorey observed, several weeks after cultured RPE cells attained confluence, the appearance of brown foci that might indicate melanogenesis occurred in cultured RPE cells.

Figure 3. Reverse transcriptase-polymerase chain reaction study of expression of various melanin related genes in cultured RPE and uveal melanoma cells. Reverse transcriptase-polymerase chain reaction products of tyrosinase, TYRP1, TYRP 2, and P gene in cultured retinal pigment epithelium cells and uveal melanoma cells were detected on agarose gels with a standard 250 bp DNA ladder as a reference for sizes (left). The four reverse transcriptase-polymerase chain reaction products products can be detected in melanoma cell line but not in retinal pigment epithelium cells.
However, porcine RPE cells were used in this experiment [44]. Pfeffer reported that human and monkey RPE cells maintained as a stable monolayer in medium containing elevated Ca\textsuperscript{2+} will undergo gradual melanization [42]. On the contrary, Rak reported that adult human RPE cells cultured with Ca\textsuperscript{2+}-switch method (low Ca\textsuperscript{2+}) expressed tyrosinase and produced melanin [43]. Champochiaro found when human RPE cells were cultured on a special extracellular matrix surface produced by bovine corneal endothelial cells, they might produce melanin [41]. None of these results have been replicated by the other researchers. If these results could be documented by a quantitative study on melanin production combined with a study on the expression of various enzymes relevant to melanogenesis, they may provide the in vitro models for studying melanogenesis of human RPE cells and could be used for various experiments that require pigmented RPE cells.

The expression of tyrosinase family genes in cutaneous melanomas depends on the types of melanoma. Melanotic tumors express tyrosinase, TRP1, and TRP2. In contrast, the amelanotic tumors lack detectable levels of one or more of these proteins. Tyrosinase was most often deficient in amelanotic tumors, whereas TRP2 was most often persistently expressed [46].

Little is known about melanogenesis of uveal melanoma cells. Several immortal human uveal melanoma cell lines (most of them are amelanotic tumors) have been established. Cells contain melanin granules and premelanosomes after numerous divisions, indicating that these cells produce melanin in vitro [24,25]. However, no quantitative studies on melanin content and melanin production in human uveal melanoma cells in vitro have been reported. Immunocytochemical studies on pathological slides of uveal melanoma found the presence of tyrosinase and TRP1 [27,28]. Several human uveal melanoma cell lines show tyrosinase activity in vitro [26]. However, there has yet to be published a systemic study on the expression of tyrosinase gene family and P gene.

In the present study, a cultured human uveal melanoma cell line (melanotic tumors) maintained a consistent level of melanin/cell and produced a detectable amount of melanin, indicating that these cells still possess melanogenic activity in vitro. RT-PCR and western blot analysis was used to demonstrate the expression of tyrosinase, TRP1, TRP2, and P gene in human uveal melanoma cells at mRNA and protein levels. Our research showed human uveal melanoma cells express genes that are essential for melanin biosynthesis in epidermal melanocytes and cutaneous melanoma cells, possibly meaning that uveal melanoma cells utilize a similar melanin biosynthesis pathway like their cutaneous counterparts.

A melanoma treatment strategy has been designed to combat tumor load using T cells that are sensitized with peptides derived from melanoma antigens, such as tyrosinase and other members of the tyrosinase family [28,46-48]. The presence of tyrosinase, TRP1, TRP2 and P protein in human uveal melanoma indicates that human uveal melanoma express these immunotherapy candidate proteins abundantly. Uveal melanoma may be a suitable candidate tumor for immunotherapeutic approaches, and tyrosinase, TRP1, TRP2, and P proteins may be the candidate proteins for immunotherapy.

In summary, this study showed adult human RPE cells do not produce melanin in vitro. The expression of four key genes involved in eumelanin biosynthesis pathway (tyrosinase, TRP1, TRP2 and P-gene) could not be detected in cultured adult human RPE cells at both mRNA and protein levels. This may explain why melanin content of RPE cells decreases significantly in the aging process and why it may be related to the occurrence of various retinal diseases, e.g. age-related macular degeneration, the leading cause of irreversible blindness among the elderly in industrialized nations. In contrast, human uveal melanoma cells still produce melanin in vitro and express all of these melanogenic genes, indicating they may possess a similar melanin biosynthesis pathway as cutaneous melanocytes and melanoma cells. Furthermore, the presence of tyrosinase, TRP1, TRP2, and P protein in uveal melanoma cells indicates that these proteins might be the candidate antigens for immunotherapy of uveal melanoma.

ACKNOWLEDGEMENTS

The authors thank Dr. G. Pelletier (Research Center of Immunology, Quebec, Canada) for providing SP6.5 melanoma cell line. This study was sponsored by The National Natural Science Foundation of China (30471857), and Zhejiang Provincial Natural Science Foundation of China (M303876 and Y204400).

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