Experimental Eye Research 128 (2014) 92–101

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Iron upregulates melanogenesis in cultured retinal pigment epithelial cells

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ARTICLE INFO

Article history:
Received 19 February 2014
Received in revised form
12 September 2014
Accepted in revised form 26 September 2014
Available online 30 September 2014

Keywords:
Iron
Retinal pigment epithelium
Human fetal retinal pigment epithelial cells
ARPE-19 cells
Melanosome
MITF
LEF 1

ABSTRACT

The purpose of our studies was to examine the relationship between iron and melanogenesis in retinal pigment epithelial cells, as prior observations had suggested that iron may promote melanogenesis. This relationship has potential clinical importance, as both iron overload and hyperpigmentation are associated with age-related macular degeneration (AMD). Human fetal retinal pigment epithelial cells and ARPE-19 cells were treated with iron in the form of ferric ammonium citrate, after which quantitative RT-PCR and electron microscopy were performed. Melanogenesis genes tyrosinase, tyrosinase-related protein 1, Hermansky–Pudlak Syndrome 3, premelanosome protein and dopachrome tautomerase were upregulated, as was the melanogenesis-controlling transcription factor, microphthalmia-associated transcription factor (*MITF*). Iron-treated cells had increased pigmentation and melanosome number. Multiple transcription factors upstream of MITF were upregulated by iron.

1. Introduction

Retinal pigment epithelial (RPE) cells are melanized cells derived from the outer layer of the optic cup neuro-ectoderm. In human RPE cells, melanosomes first appear during the 7th week of gestation (Breathnah and Wyllie, 1966, Hogan et al., 1971, Marmor et al., 1998, Mund et al., 1972). Like melanosomes in other pigmented cells of the body, RPE melanosomes undergo a four stage maturation process which can be observed ultrastructurally (Feeney-Burns, 1980, Marmor et al., 1998, Nordlund, 2006). In Stage I, vesicles are formed (Nordlund, 2006). In Stage II, a protein called premelanosome protein (PMEL) assembles into a scaffold of amyloid-like fibrils, creating a striated appearance (McGlinchey et al., 2011, Nordlund, 2006). In Stage III, proteins important for melanin synthesis, such as tyrosinase (TYR), tyrosinase related protein (TYRP1), and dopachrome tautomerase (DCT), are transported to the melanosome and begin to deposit melanin on the PMEL fibrils (Nordlund, 2006). In Stage IV, the melanosome is completely melanized, so that the fibrils are no longer visible (Nordlund, 2006).

In the human RPE cell a mixture of all four stages of melanosomes is seen between fetal weeks 7–14 (Marmor et al., 1998, Stroeva and Mitashov, 1983). Generation of new melanosomes then stops, and the already formed melanosomes continue maturing (Stroeva and Mitashov, 1983). By 27 weeks gestation, all of the melanosomes are melanized and are Stage IV granules (Stroeva and Mitashov, 1983). Although the melanosomes have matured to Stage IV by fetal week 27, melanin continues to be deposited in them until the age of 2 years (Marmor et al., 1998, Stroeva and Mitashov, 1983). It has long been held that no new melanosomes are synthesized in the RPE cells after the age of 2 years in humans; however, some argue that there is a low level of

Abbreviations: hfRPE cells, Human Fetal Retinal Pigment Epithelial Cells; FAC, Ferric Ammonium Citrate.

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http://dx.doi.org/10.1016/j.exer.2014.09.010
0014-4835/

melanosome turnover and melanogenesis in adult life (Schraermeyer and Heimann, 1999).

Melanosomes within RPE cells serve multiple roles. They aid in visual function by absorbing and preventing the backscattering of light (Land et al., 2004, Marmor et al., 1998), they play a role in the development of the visual system (Guillery, 1996, Marmor et al., 1998), they bind metals, including calcium, zinc, copper and iron (Hong and Simon, 2007, Kaczara et al., 2012, Rozanowski et al., 2008), and they scavenge free radicals (Rozanowska et al., 1999, Rozanowski et al., 2008). With age, RPE cells accumulate lipofuscin granules containing pro-oxidant compounds such as A2E, which contribute to cell ageing and damage (Sparrow and Boulton, 2005). It is thought that by binding free radicals and metals, the melanosomes may protect the RPE cells from reactive oxygen species and may enhance lysosomal function in RPE cells (Rozanowski et al., 2008). Although intact melanosomes act as anti-oxidants, photodegraded melanosomes may become pro-oxidant, likely by releasing the metals that they were binding (Rozanowski et al., 2008). In certain diseases, such as age-related macular degeneration and aceruloplasminemia where RPE cells contain increased levels of iron (Hahn et al., 2003, Wolkow et al., 2011), melanosomes may initially play a protective role by sequestering iron. However, as iron can cause physical damage to melanosomes and contribute to their degradation (Korytowski and Sarna, 1990, Wolkow et al., 2011). Over time melanosomes may degenerate, releasing iron and contributing to disease progression.

The relationship between melanosomes and iron may extend beyond the physical effects of iron on melanin, as iron may also alter signaling pathways that contribute to melanosome biosynthesis and degradation. For example, when a microarray analysis was performed on the RPE cells of $Cp^{-/-}$, $Heph^{sla/sla}$ mice, which accumulate iron in the RPE cells, the gene with the most significant changes was Hermansky–Pudlak Syndrome 3 (*HPS3*) (Dunaief lab, unpublished data). HPS3 is part of BLOC (biogenesis of lysosome-related organelles complex), which sorts and traffics proteins such as tyrosinase (TYR) and tyrosinase-related protein-1 (TYRP1) to developing melanosomes (Di Pietro et al., 2004, Raposo and Marks, 2007). Similarly, microarray studies on mice with mutations in the master melanogenesis transcription factor, microphthalmia-associated transcription factor (MITF), have demonstrated changes in the iron transporter *Nramp1* (*Slc11a1*) in RPE cells (Gelineau-van Waes et al., 2008).

Additional evidence suggests that iron homeostasis is linked to melanogenesis. Patients with iron overload, as well as with overload by other metals, have increased skin pigmentation resulting from increased melanin levels (Nordlund, 2006). In the disease hemochromatosis, where multiple organs are overloaded with iron, the darkening of skin is due to increased melanin levels and not to iron deposition (Cawley et al., 1969, Nordlund, 2006, Perdrup and Poulsen, 1964). Although a link between iron and melanogenesis has been observed, the mechanism for this association has not been previously described (Cawley et al., 1969, Nordlund, 2006). The purpose of our current studies was to examine the relationship between iron and melanogenesis in the RPE at the molecular level.

2. Materials and methods

2.1. Cell culture

2.1.1. Human fetal retinal pigment epithelial (hfRPE) cell culture

Eyes from 16 to 19 week old donors were obtained from Advance Bioscience Resources (Alameda, CA), and cultures were established as previously described (Maminishkis et al., 2006, Maminishkis and Miller, 2010). Briefly, eyes were rinsed in antibiotic–antimycotic plus gentamicin solution (Invitrogen, Grand Island, NY), and eyecups were made. Eyecups were incubated in dispase-I (Roche Diagnostics, Indianapolis, IN) solution in 5% serum hfRPE media for 30 min (Maminishkis et al., 2006, Maminishkis and Miller, 2010). Single cell RPE layers were peeled off in sheets and collected in 5% hfRPE medium. Cells were washed, separated into single cells and put into T25 Primaria flasks (Fisher Scientific, Pittsburgh, PA) in 15%-serum containing media (Maminishkis et al., 2006, Maminishkis and Miller, 2010). After one day, media was changed to 5% serum hfRPE media. Media was then changed three times per week. After at least 4 weeks in T25 flasks, the hfRPE cells were trypsinized with 0.25% trypsin (Invitrogen, Grand Island, NY) and seeded onto cell culture transwell inserts (cat 3460, Corning-CoStar, Corning, NY) that were coated with human extracellular matrix (cat 354237, BD Biosciences, San Diego, CA), as previously described (Maminishkis et al., 2006, Maminishkis and Miller, 2010). Cells from 7 different donors were used to establish the cultures used for these experiments, they were grown for at least 4 weeks on transwell inserts prior to use. All cells used in experiments had a transepithelial resistance of $>300\ \Omega\ cm^2$.

2.1.2. ARPE-19 cell culture

ARPE-19 cells obtained from ATCC (Manassas, VA) and were grown as previously described (Dunn et al., 1996). Cells from passages 24–29 were used for current experiments. Confluent flasks were split in a 1:4 ratio. Freshly passaged ARPE-19 cells were allowed to reach full confluence in high serum media D10-F12, after which they were switched to iron treatment conditions. Cells were maintained at confluence without further splitting until the end of the experiment. Cells were treated with or without 250 μM FAC for one week or 2 months as described below. Cells in high serum conditions were maintained in high glucose D10-F12 media during the experiment (the 10 in D10 refers to 10% Fetal Bovine Serum, and the DMEM had 4.5 g/L glucose), while cells in low serum conditions were grown in low glucose D1-F12 media (the 1 in D1 refers to 1% Fetal Bovine Serum, and the DMEM had 1 g/L of glucose).

2.1.3. Treatment with Ferric Ammonium Citrate

All cells were treated with 250 μM Ferric Ammonium Citrate (Sigma–Aldrich, St. Louis, MO) for 1 week or 2 months. Prior to each treatment, fresh 50 mM ferric ammonium citrate stock solution was made in 5% hfRPE media, D1-F12 media (containing 1% serum), or D10-F12 media (containing 10% serum) and sterile filtered. The stock solution was further diluted to 250 μM in 5% hfRPE media, D1-F12, or D10-F12.

2.2. Quantitative RT-PCR analysis

RNA was extracted from treated cells using the QIAGEN RNeasy Mini Kit and QIAshredder Spin columns (Valencia, CA) as described by the manufacturer. Reverse transcription was performed using the Taqman Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA). For quantitative PCR all materials and reagents were obtained from Applied Biosystems (Carlsbad, CA) and reactions were run on a 7500 Fast Real-Time PCR System Thermocycler (Applied Biosystems, Foster City, CA). The following probes from Applied Biosystems were used: human Eukaryotic 18S rRNA (Hs99999901_s1), *TFRC* Hs00174609_m1, *TYR* Hs01099965_m1, *TYRP1* Hs00167051_m1, *HPS3* Hs00289968_m1, *DCT* Hs01098278_m1, *PMEL* Hs00173854_m1, *MITF* Hs01117294_m1, *DICER1* Hs00229023_m1, *XPC* Hs01104206_m1, *PAX3* Hs00240950_m1, *SOX10* Hs00366918_m1, *SOX9* Hs00165814_m1, *LEF1* Hs01547250_m1, *OTX2* Hs00222238_m1 and *NRF2/NFE2L2* Hs00975961_g1. For fRPE cells, triplicate culture wells from each of