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Studying melanin and lipofuscin in RPE cell culture models

Michael E. Boulton*

Department of Ophthalmology, Indiana University School of Medicine, USA

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ABSTRACT

The retinal pigment epithelium contains three major types of pigment granules; melanosomes, lipofuscin and melanolipofuscin. Melanosomes in the retinal pigment epithelium (RPE) are formed during embryogenesis and mature during early postnatal life while lipofuscin and melanolipofuscin granules accumulate as a function of age. The difficulty in studying the formation and consequences of melanosomes and lipofuscin granules in RPE cell culture is compounded by the fact that these pigment granules do not normally occur in established RPE cell lines and pigment granules are rapidly lost in adult human primary culture. This review will consider options available for overcoming these limitations and permitting the study of melanosomes and lipofuscin in cell culture and will briefly evaluate the advantages and disadvantages of the different protocols.

1. Introduction

In mammals, cells derived from both the neural crest and neuroepithelium participate in melanogenesis (Rozanowska, 2011; Sarna, 1992). Melanogenesis in melanocytes derived from the neural crest results in the melanin observed in the uveal melanocytes of the iris and choroid. By contrast, the ciliary body, iris, and the retinal pigment epithelium are derived from the neuroepithelium. The first meaningful descriptions of a pigmented layer, distinct from the choroid, in the human eye was provided by the Mondini family at the end of the 18th century and it wasn't until 1833 that the first paper correctly describing retinal pigment epithelium (RPE) histology in humans and a number of other mammals was published by Thomas Wharton Jones (reviewed in Wolfensberger, 1998). Subsequent studies have identified three major types of pigment granule in the human RPE: melanosomes formed during a brief window of embryogenesis, lipofuscin granules that accumulate with increasing age and melanolipofuscin granules which are a feature of aged RPE (Feeney, 1978). Melanosomes have been most studied in the context of development and melanogenesis while lipofuscin has been extensively studied as it is considered to have a strong association with the pathogenesis of age-related macular degeneration (AMD). While reproducible cell culture protocols for human RPE were introduced in the 1980's a notable observation was the loss of pigment granules (both melanosomes and lipofuscin) through their repeated dilution amongst daughter cells (Boulton et al., 1983; Flood et al., 1980). While melanogenesis is observed in fetal RPE cultures (Hu and Bok, 2001; Maminishkis et al., 2006) and both embryonic stem cell – and induced pluripotent stem cell – derived RPE (Rowland et al., 2013; Vugler et al., 2008) it is not routinely observed in cultures from adult donor RPE (Boulton, 1998) in which key enzymes in the melanogenesis pathway are no longer expressed (Lu et al., 2007). Furthermore, since a) the melanogenesis pathway was defined decades ago (reviewed in Boulton, 1998), b) melanin has not been shown to be strongly associated with major retinal diseases such as AMD, DR and inherited retinal degenerations and c) the more popular mammalian RPE cell lines (e.g. ARPE19, D407, RPE-J) do not readily demonstrate melanogenesis the study of melanin and the formation of melanin granules in cultured RPE cells has not been extensive. By contrast, for lipofuscin, there have been a large number of studies and numerous protocols exist that generate autofluorescent granules with some similarities to lipofuscin but none that fully recapitulate the biophysical features of bona fide human RPE lipofuscin (Boulton, 2009; Sparrow and Boulton, 2005). The following sections will focus on mammalian RPE

* Department of Ophthalmology, Indiana University School of Medicine, 930 Walnut Street, R3 – C504, Indianapolis, IN 46202, USA.
E-mail address: mboulton@iupui.edu.

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cells, primarily human, and critically discuss protocols to study pigmentary processes in the adult RPE.

2. Melanosomes in cultured RPE cells

2.1. Melanogenesis, a developmental perspective

Melanogenesis in mammals occurs during a brief window of embryogenesis and thereafter new melanin is formed at a very low level, if at all (reviewed in Boulton, 1998; Sarna, 1992). In man melanin granules are usually observed between 27 and 30 days of fetal development and by the 14th week melanosomes at all stages of maturation can be observed (Rozanowska, 2011). Melanogenesis then stops within a few weeks as the cells reach their full complement of pigment granules. Melanosomes mature through a number of stages and can generally be ascribed as follows (Boulton, 1998; Riley, 1980; Rozanowska, 2011). First, a non-pigmented, poorly defined ovoid protein matrix is formed in the smooth endoplasmic reticulum and released into the cytosol where tyrosinase (a critical enzyme in melanin formation) is transferred via vesicular transport from the Golgi and melanin synthesis begins (Boulton, 1998). The stage I melanosome, or premelanosome, assumes a structured protein matrix on which melanin (predominantly eumelanin) is deposited. Melanin deposition and granule maturation continue for up to two years in man until fully melanized, stage IV melanosomes are observed. It is important to note the following in terms of melanosome formation in the RPE: a) tyrosinase is a rate limiting enzyme that converts L-tyrosine to dopaquinone and that in the absence of tyrosinase activity no melanin is formed and b) tyrosinase is only synthesized in postnatal RPE and not adult RPE (Rozanowska, 2011; Sarna, 1992). Melanin granules are abundant in the adult human RPE and are predominantly located in the apical and mid-portions of the cell. It is important to note that there is regional distribution of melanosomes in mammalian eyes with darker pigmentation of RPE at the macula in human eyes and complete absence of melanosomes in areas of the RPE in eyes of animals with a tapetal fundus (Boulton, 1998). Furthermore, melanosomes undergo significant age-related changes through a combination of photochemical modification and lysosomal degradation which changes their spectral characteristics and increases their photoreactivity and potential for phototoxic damage to the RPE (Boulton et al., 1990; Rozanowski et al., 2008). In addition, melanolipofuscin granules appear later in life and represent a complex of both melanin and lipofuscin. The synthesis of melanin and the formation of melanosomes within the adult RPE remain controversial and are discussed further below.

2.2. Melanogenesis in adult RPE cell cultures

Despite the acceptance that melanogenesis occurs in the RPE during a brief window of embryogenesis and thereafter the pathway is switched off. mRNA and protein expression for critical components of the melanogenesis pathway (tyrosinase, tyrosinase-related proteins 1 and 2 and P-gene) are not detected in cultured adult human RPE (Lu et al., 2007) and there have been numerous reports of repigmentation in ARPE19 and adult primary RPE cells (Ahmado et al., 2011; Campochiaro and Hackett, 1993; Dorey et al., 1990; Gamm et al., 2008; Koh and Kane, 1992; Liu et al., 2009; Mannermaa et al., 2010; Ohno-Matsui et al., 2005; Rak et al., 2006; Smith-Thomas et al., 1996; Stanzel et al., 2005). When pigmented primary human RPE cells are placed in primary culture they rapidly depigment due to the sharing of pigment granules between daughter cells (Boulton et al., 1983; Flood et al., 1980). However, both extracellular matrix and calcium have been reported to promote repigmentation, although it must be emphasized that there was scant use of ultrastructural examination or compositional analysis to confirm that these were bona fide melanosomes. However, some studies did show an increase in melanogenesis related genes (Mannermaa et al., 2010). A number of studies have shown that matrix can influence repigmentation in primary RPE cultures. Matrices include corneal endothelial matrix (Campochiaro and Hackett, 1993), laminin + fibroblast growth factor (Campochiaro and Hackett, 1993; Smith-Thomas et al., 1996), cell culture inserts (Mannermaa et al., 2010) and amniotic membrane (Ohno-Matsui et al., 2005; Stanzel et al., 2005).

Rak and colleagues used a calcium switch protocol whereby sixth passage depigmented primary human RPE cells were suspended in calcium-free medium and calcium levels then increased to 50 μM (Rak et al., 2006). These cells showed tyrosinase expression and by 8 weeks in culture began to demonstrate pigmentation while these characteristics were not observed in control cells. ARPE19 cells cultured on filter inserts for up to 3 months in DMEM medium with 1 mM pyruvate generated “dark pigmentation” which was not observed in the absence of pyruvate (Ahmado et al., 2011). Vasoactive intestinal peptide has been reported to promote pigmentation in chick RPE cells (Koh and Kane, 1992). A few groups have reported that tyrosinase (the rate limiting enzyme in melanogenesis) can be induced in postnatal RPE by phagocytosis of rod outer segments (Schraermeyer et al., 2006), orthodenticle homeobox 2 (OTX2) activation of the human tyrosinase gene promoter (Reinisalo et al., 2012), overexpression of tyrosinase (Biesemeier et al., 2010) or overexpression of the transcriptional repressor Zeb1 (Liu et al., 2009) and that these cells show increased pigmentation. It is possible that melanin formation and deposition can occur via non classical pathways within multivesicular bodies in the absence of premelanosomes (Biesemeier et al., 2010). The hTERT-RPE immortalized human RPE cell line is reported to undergo spontaneous melanization within 4 weeks in culture (Rambhatla et al., 2002).

The problem is that true melanogenesis as described in section 2.1 has not been unequivocally demonstrated in adult RPE cultures. Identifying electron dense structures in RPE cells is not sufficient. It is recommended that investigators confirm melanogenesis and melanosome formation using a combination of transmission electron microscopy, gene expression analysis for genes associated with the melanogenesis pathway and use chemical assays to confirm the accumulation of melanin. Since ARPE19 cells lack melanin but recapitulate most of the biological functions attributed to RPE cells *in vivo*, the extent to which melanosomes augment facets of RPE differentiation or function is likely to be modest. Interestingly, RPE cells derived from bone marrow cells are poorly pigmented *in vitro* but when transplanted into the retina they rapidly repigment with typical melanosomes emphasizing the importance of the niche environment (Sengupta et al., 2009).

2.3. Melanogenesis in fetal RPE cell cultures

Bok and colleagues reported the generation of highly differentiated and polarized human RPE cultures derived from fetal donors (Frambach et al., 1990; Hu and Bok, 2001). Fetal human RPE cells were initially grown to confluence in low-Ca^{++} Chee’s essential modified medium (CEM) until cells were released into the medium. These nonattached cells were collected and subsequently cultured for 4 months to 2 years on culture inserts using a 1:1 mixture of normal-calcium CEM and Eagle’s minimum essential medium with 1% heat-inactivated calf serum. The authors reported pigment granules that were polarized toward the apical region within the cells with extended culture time (Hu et al., 1994) but electron microscopy did not show typical melanosome structures and there was no attempt to document melanogenesis or analyze melanin

type or content. Subsequently, Maminishkis et al. (2006) reported a modification of this approach using a specifically designed medium which allowed freshly isolated fetal RPE cell to be grown on cell culture flasks. Pigment density was observed to initially decrease through dilution amongst daughter cells, as is observed in adult RPE cultures, but as the cells became confluent de nova synthesis of pigment was observed and by 30 days pigment density was comparable to that observed in native RPE cells. Electron microscopy demonstrated the presence of characteristic melanosomes that were located in that apical portion of the cell. Despite the potential to further study RPE melanogenesis in these fetal-derived cultures reports in melanization are sparse. Reasons for this are likely to include the complex isolation procedures involved together with the culture duration of 28+ days to achieve pigmentation.

2.4. Melanogenesis in stem cell-derived RPE cells

There is a wealth of literature detailing the successful differentiation of human embryonic stem cells (hESC) and induced pluripotent stem cells into RPE cells (reviewed in Carr et al., 2013; Rowland et al., 2012). It is not the role of this review to discuss culture conditions but it is important to note that these derived RPE cells all exhibit an RPE phenotype including pigment granules (Rowland et al., 2013; Sorkio et al., 2014; Subrizi et al., 2012). This suggests that these cells may retain the characteristics of fetal RPE cells. Due to the complex procedures involved and the time taken (up to 3–4 months) the efficiency of this model for studying melanization is limited. Bone marrow-derived cells can, under the appropriate conditions differentiate into RPE-like cells which contains melanosomes (Harris et al., 2006; Sengupta et al., 2009). However, while these cells form typical RPE cells when injected *in vivo* they do not polarize or differentiate well in culture suggesting the importance of the *in vivo* niche in the differentiation process. Retinal stem cells derived from adult ciliary body have been reported to differentiate into a functional polarized RPE which after 7 days, to show different stages of melanogenesis and by 15 days in culture only mature melanosomes are observed (Aruta et al., 2011). Recently, Salero et al. (2012) demonstrated a subpopulation of adult human RPE cells can be activated *in vitro* to a self-renewing cell, the retinal pigment epithelial stem cell (RPESC) that can redifferentiate into stable cobblestone RPE monolayers and exhibit some level of pigmentation although this was not well defined.

2.5. Repigmentation with mature melanosomes from donor eyes

An alternative option is to repigment cultured RPE cells with intact pigment granules isolated from ex vivo RPE cells. An advantage of this approach is that the cells now contain intact mature melanosomes with the same properties as those *in vivo* which take into consideration age-related changes reported for melanin granules (Boulton et al., 1990; Rozanowski et al., 2008). Human melanosomes from the preferred age group are isolated and purified from donor eyes as previously described (Boulton and Marshall, 1985; Rozanowski et al., 2008). In brief, the neural retina is detached from the underlying RPE and the RPE cells are detached from Bruch's membrane using a fine brush and the cells are then disrupted by mechanical homogenization. After removal of the debris the melanosomes are purified on a discontinuous sucrose gradient (1–2 M) at 103,000 g. Melanosomes are sufficiently dense that they pass through the gradient and form a pellet. After resuspension and washing, the melanosomes are suspended in culture medium and added to confluent cell cultures (preferably polarized) at about ~300 granules per cell for 24 h to allow the granules to be phagocytosed where they end up in lysosomes similar to that seen *in vivo*. It is advisable to allow the cells to stabilize in basal medium for 7–14 days prior to undertaking experiments. This approach works in both primary human RPE cultures and cell lines such as ARPE19.

This repigmentation approach allowed Rozanowski and colleagues to demonstrate that melanosomes can be phototoxic to primary human RPE cells and supported a contributing role of melanosomes in RPE aging (Rozanowski et al., 2008). In this study cells with and without repigmentation with melanosomes were either maintained in the dark or exposed to blue light for up to 96 h and assessed for alterations in cell morphology, cell viability and lysosomal integrity. Exposure to blue light in the presence of melanosomes from old donors resulted in abnormal cell morphology, up to approximately 75% decrease in mitochondrial activity, loss of lysosomal pH and cell death while this was not observed for melanosomes isolated from young donors.

An alternative source of melanosomes is bovine or porcine eyes but these granules normally represent newly formed melanosomes as the cattle are slaughtered around 12 months of age and therefore do not model the age-related changes that are associated with melanosomes (Boulton, 1998; Sarna, 1992). However, these granules when ingested by RPE cells provide an excellent model for studying melanosome–iron interactions (Kaczara et al., 2012), melanosome mobility (Burke and Zareba, 2009), the cytoprotective capacity of melanosomes (Burke et al., 2011; Zareba et al., 2006), Influence of pigment content, intracellular calcium and cyclic AMP on the ability of human primary RPE cells to contract collagen gels (Smith-Thomas et al., 2000) and the capacity of melanosomes to bind drugs (Basu et al., 1989). To better mimic the aged RPE investigators have "aged" the melanosomes by either photochemical change or oxidation prior to feeding them to RPE cells. Such studies have shown that these modified granules increase RPE cell photic stress compared to untreated melanosomes (Zareba et al., 2007).

3. Lipofuscin accumulation in cultured RPE cells

3.1. Lipofuscinogenesis

The accumulation of lipofuscin granules within lysosomes is a hallmark of a variety of highly metabolically active post mitotic cells (reviewed in Boulton, 2009; Sparrow and Boulton, 2005). In the human RPE, lipofuscin accumulates progressively throughout the first 6 decades of life to occupy up to 19% of cytoplasmic volume but thereafter levels tend to plateau. Excessive accumulation of lipofuscin within the RPE has been associated with a variety of retinal disorders including: age-related macular degeneration, retinitis pigmentosa, Stargardt's disease and Best's disease. The genesis and composition of lipofuscin granules within the RPE remains an area of considerable debate. RPE lipofuscin is believed to result from the incomplete degradation of both phagocytosed photoreceptor outer segments and autophagic removal of damaged organelles/protein aggregates (Boulton, 2009; Boulton et al., 1989; Burke and Skumatz, 1998). However, the relative contribution of these two pathways remains unknown as does the composition of the resultant granules. Despite over 2 and a half decades of research we still only have a limited understanding of the composition of lipofuscin and this has in part been hampered by contamination of isolated granules with other intracellular material. Eldred and Katz first identified the bis-retinoid, N-retinylidene-N-retinylethanolamine (A2E) in a chloroform extract of lipofuscin (Eldred and Katz, 1988). A2E has subsequently received extensive attention both as a marker for lipofuscin and as a potentially toxic component of lipofuscin granules (Sparrow and Boulton, 2005; Sparrow et al., 2012). However, as will be discussed below, the role of A2E may be much less impactful than originally thought (Smith et al., 2013).

Surprisingly, purified lipofuscin granules contain little or no protein but do contain significant amounts of modified material such as carboxyethylpyrrole adducts suggesting they consist largely of non-polar components (Ng et al., 2008).

3.2. Photoreceptor-derived autofluorescent granules

Lipofuscin in the RPE is considered to be predominantly derived from the incomplete digestion of photoreceptor outer segments (POS). Over the past two and a half decades there have been a plethora of studies reporting that repeated phagocytosis of isolated photoreceptor outer segments leads to the progressive accumulation of autofluorescent granules within cultured primary RPE cells and RPE cell lines (Boulton et al., 1989; Guha et al., 2013; Kaemmerer et al., 2007; Lei et al., 2013; Nilsson et al., 2003; Thampi et al., 2012; Wihlmark et al., 1996). The first such report was by Boulton and colleagues who fed cultured primary human RPE cells purified bovine rod outer segments daily for up to 3 months (Boulton et al., 1989). They estimated that each cell was exposed to ~200 outer segments per day, which is significantly greater than the estimated 40 photoreceptor outer segments associate with each RPE cell *in vivo*. However, they did not measure how many outer segments were ingested per cell. Autofluorescent granules were observed within 2 weeks of daily feeding with POS and the number of granules increased until the study was terminated at 3 months. Although standard fluorescence microscopy and electron microscopy demonstrated granules with all the appearance of lipofuscin they did not exhibit identical spectral and compositional characteristics when compared to ex vivo human bona fide RPE lipofuscin granules (Wassell et al., 1998). The spectral emission characteristics of these granules tended to be in the blue region with peak emission at 425 and 525 nm while lipofuscin granules showed a characteristic broad band emission with peaks at 560, 600 and 625 nm. Thin layer chromatography of the chloroform soluble fraction from these granules showed only blue yellow and green fluorophores, but none of the characteristic yellow orange associated with true RPE lipofuscin. Thus, while POS-fed cultured RPE cells may provide a model for generating autofluorescent granules, caution should be taken in concluding that the properties of these *in vitro* granules truly reflect the biophysical properties of RPE lipofuscin. Interestingly, cells accumulating these POS-derived autofluorescent granules are considerably more sensitive to visible blue light than unloaded control cells and show lysosomal membrane destabilization and loss of cell viability (Brunk et al., 1995; Wihlmark et al., 1997). The predominant blue-emitting fluorophores in the cell culture-derived autofluorescent granules probably consists of cross-linked products of lipid peroxidation, proteins and carbohydrates (Wassell et al., 1998). There is a body of literature that proposes RPE lipofuscin results from the incomplete degradation of oxidatively modified POS. It has been demonstrated that RPE cells exposed to peroxidized POS accumulated significantly more autofluorescent material than those exposed to native POS, supporting the view that peroxidized outer segments are less digestable by lysosomal enzymes (Brunk et al., 1995; Wihlmark et al., 1996).

3.3. Autophagy-derived autofluorescent granules

Autophagy is a key process in maintenance of cellular homeostasis that serves to remove dysfunctional organelles and proteins. The age-related increase in lipofuscin accumulation that occurs in a wide variety of highly metabolically active, post mitotic cells throughout the body is thought to a result of the autophagic removal of damaged organelles and their transfer to the lysosomal system for degradation. There is now considerable evidence that the RPE, like most other cells, maintains basal autophagy for cellular homeostasis and that this changes with both age and disease (Kaarniranta et al., 2013; Krohne et al., 2010; Mitter et al., 2012). It is often ignored that autophagy contributes significantly to the accumulation of lipofuscin and this may, in part, explain granule heterogeneity (Clancy et al., 2000). Numerous studies have shown that cultured RPE cells accumulate autofluorescent granules with many of the features of lipofuscin in the absence of a photoreceptor substrate, strongly suggestive that autophagy is involved (Boulton et al., 1989; Burke and Skumatz, 1998; Krohne et al., 2010; Kurz et al., 2009). Burke and Skumatz (1998) demonstrated that autofluorescent inclusions accumulated in human and bovine primary RPE cultures after extended post-confluent periods. In these studies first-passage human RPE cultures were grown to confluence and then passaged as many as 18 times at a split ratio of approximately 1:3. Autofluorescence was examined in cultures in various passages that were maintained undisturbed at confluence for intervals from 1 week to 2 years. The accumulation of autofluorescent material was not increased in aged versus young donors or with increased passage. The number of granules per RPE cell varied for lipofuscin *in situ* and inclusions *in vitro*, although the latter were more heterogeneous in size and shape and in ultrastructural appearance of the granule contents. Wassell et al. (1998) made a similar observation but in this case cells were maintained in basal medium (Ham's F-10 + 2% fetal calf serum) for up to 56 days with the medium replaced every 3 days. There was a significant accumulation of autofluorescent granules which could be further enhanced by inhibition of the protease inhibitor leupeptin which was presumed to prevent the removal and degradation of material contained within autophagosomes. The accumulation of these granules was always less than that achieved by feeding the RPE cells POS. Similar to results obtained from repeatedly feeding cultured RPE cells POS, the autophagic-derived autofluorescent granules did not exhibit identical spectral and compositional characteristics when compared to RPE lipofuscin granules nor were the spectral emission characteristics of these granules the same (Wassell et al., 1998). However, that these autofluorescent granules show at least some of the spectral and compositional characteristics of lipofuscin suggests that the autophagic pathway can make a significant contribution to lipofuscin accumulation in the RPE *in vivo* (Mitter et al., 2012). Of major importance is the observation that these autofluorescent granules are highly phototoxic to cultured RPE cells when exposed to blue light further emphasizing that retinoids and their metabolites are not the sole, or even the major source of photoreactivity in lipofuscin granules (Boulton, 2009). A number of *in vitro* studies have been undertaken to assess the relative contribution of autophagy-derived and POS-derived autofluorescent granules but results must be interpreted cautiously given the recent report that microtubule-associated protein 1A/1B-light chain 3 (LC3), a protein thought to be restricted to the autophagic pathway is also implicated in RPE phagocytosis (Kim et al., 2013).

3.4. Adding A2E to RPE cultures

A2E is a bisretinoid that is the product of all-*trans* retinal and etholamine and which has long been considered a major fluorophore and biomarker of lipofuscin (Sparrow and Boulton, 2005; Sparrow et al., 2012). However, a recent study has questioned this and reported a lack of correlation between the spatial distribution of A2E and lipofuscin fluorescence in the human RPE (Ablonczy et al., 2013). Thus, for this and reasons described below, exogenous A2E may well not represent a good model for elucidating the role of lipofuscin in the RPE of retinal diseases such as AMD. However, it is possible that other bisretinoids or their

photodegradation products may play a more prominent role in retinal pathologies such as AMD (Sparrow et al., 2012).

A typical protocol for the investigation of A2E in both primary RPE cell cultures and the ARPE19 cell line involves exposure of cells to concentrations of synthesized A2E dissolved in dimethyl sulfoxide at concentrations ranging from 10 to 100 μM at which A2E does not exert any overt toxicity on RPE cells (Sparrow et al., 2000; Vives-Bauza et al., 2008). A2E is lysosomotropic compound that localizes predominantly to lysosomes and does not reach significant levels in other cellular membranes including mitochondria, Golgi and endoplasmic reticulum (Schutt et al., 2000; Sparrow et al., 2000; Vives-Bauza et al., 2008). Exposure of RPE cultures to 50 μM A2E results in lysosomal A2E levels similar to those of human normal subjects of old age (Vives-Bauza et al., 2008). An alternative approach has been to complex A2E with low density lipoprotein as a transport vehicle to the lysosomes (Schutt et al., 2000). It is normal to let the cultures stabilize for 7–14 days after A2E treatment. Numerous studies have shown that exposure of A2E fed cells mediates blue light-induced damage to RPE cells leading to apoptotic cell death (Schutt et al., 2000; Sparrow et al., 2000). It is likely that this occurs through the photogeneration of singlet oxygen although an alternative approach is that A2E is itself oxidized to generate more damaging intermediates. Other effects ascribed to A2E in cell culture systems include a) elevation of lysosomal pH and reduced degradative capacity (Holz et al., 1999), b) reduced mitochondrial membrane potential and inhibited oxidative phosphorylation of RPE cells (Vives-Bauza et al., 2008), c) inhibition of phagocytosis (Vives-Bauza et al., 2008), d) activation of the complement pathway, e) promoting the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) (Zhou et al., 2005), f) inducing interleukin-1β (IL-1β) production in RPE cells via the NLRP3 inflammasome (Anderson et al., 2013) and g) indirectly modifying proteins by advanced glycation endproduct formation by generating glyoxal and methylglyoxal (Yoon et al., 2012).

Application of exogenous A2E has become a model of convenience for those studying lipofuscin and AMD but the relevance of A2E to the cytotoxicity of lipofuscin is open to question (Boulton, 2009). A2E is only weakly photoreactive in comparison to mature lipofuscin granules and low levels of A2E are reported to protect against oxidative damage. A2E only represents a small proportion of visible light absorbed by lipofuscin granules. Autophagic-derived lipofuscin granules devoid of retinoids are highly photoreactive and capable of causing cell death. Furthermore, there is no evidence that A2E can be released once complexed into lipofuscin granules and thus it is unlikely to be present in its free form at high levels. In conclusion, data from *in vitro* A2E studies may represent phenomenology rather than a true reflection of the properties of mature lipofuscin granules.

3.5. Repigmentation with mature lipofuscin granules from donor eyes

As discussed earlier for melanosomes, it is easy to similarly repigment cultured RPE cells with intact lipofuscin granules isolated from ex vivo RPE cells. An advantage of this approach is that the cells now contain intact mature lipofuscin granules that reflect the *in vivo* situation rather than treating cells with constituents of lipofuscin or inducing the accumulation of autofluorescent material in RPE cultures. Human lipofuscin granules are isolated and purified from donor human eyes as described above for melanosomes with the exception that lipofuscin granules sediment at the 1.55/1.6 M interface of the sucrose gradient (Boulton and Marshall, 1985; Davies et al., 2001). After resuspension and washing, the lipofuscin granules are suspended in culture medium and added to confluent cell cultures (preferably polarized) at about ~300 granules per cell for 24 h to allow the granules to be phagocytosed where they end up in lysosomes similar to that seen *in vivo*. It is advisable to allow the cells to stabilize in basal medium for 7–14 days prior to undertaking experiments.

This lipofuscin repigmentation model was the first to demonstrate the phototoxic potential of lipofuscin in the retina (Davies et al., 2001). Primary human RPE cultures fed isolated lipofuscin granules or unfed control cultures were either maintained in the dark or exposed to visible light for up to 48 h. Exposure of lipofuscin-fed cells to short wavelength visible light (390–550 nm) caused lipid peroxidation (increased levels of malondialdehyde and 4-hydroxy-nonenal), protein oxidation (protein carbonyl formation), loss of lysosomal integrity, cytoplasmic vacuolation, and membrane blebbing culminating in cell death. This effect was wavelength-dependent because light exposure at 550–800 nm had no adverse effect on lipofuscin-loaded cells. These results confirmed the phototoxicity of lipofuscin in a cellular system and implicated it in cell dysfunction such as occurs in ageing and retinal diseases. Furthermore, exposure of lipofuscin-containing cells to visible light also causes an increase in both mitochondrial and nuclear DNA lesions compared with non-pigmented cells (Godley et al., 2005).

4. Conclusions

There are many *in vitro* models described to study the genesis, role and consequences of pigment in the RPE. While these models can be ideal for studying mechanism, caution should be applied when trying to relate them to retinal pathology. Melanosome formation, replicating that seen during embryogenesis, is easy to achieve in fetal RPE cells and stem-cell derived RPE. However, this recapitulates a phenotype associated with the young and does not provide a model for age-related changes in melanosomes or the consequences of these changes in retinal dysfunction. Melanogenesis in adult RPE cultures is equivocal and interpretation of such data should be carefully considered. Understanding lipofuscin and its contribution to retinal pathology has gained much from RPE cell culture techniques using ex vivo granules and material generated from the incomplete degradation of phagocytosed outer segments or autophagy of intracellular organelles. However, the plethora of data generated with the bis-retinoid A2E in RPE cells now needs to re-evaluated following the observation that it is not prominent in the central retina. In conclusion, there are numerous RPE culture models available to study pigmentation and choice is dependent on the hypothesis to be tested and the outcome measures required.

Conflict of interest

The author has no conflict of interest.

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