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α v β 5 INTEGRIN RECEPTORS AT THE APICAL SURFACE OF THE RPE: ONE RECEPTOR, TWO FUNCTIONS

Emeline F. Nandrot¹, Yongen Chang¹, and Silvia C. Finnemann^{1,2}

¹Margaret M. Dyson Vision Research Institute, Department of Ophthalmology, Weill Medical College of Cornell University, Box 233, 1300 York Avenue, New York, NY10021, USA

²Department of Cell and Developmental Biology, and Department of Physiology and Biophysics, Weill Medical College of Cornell University, Box 233, 1300 York Avenue, New York, NY10021, USA

1. INTRODUCTION

Photoreceptors and retinal pigment epithelial (RPE) cells, two adjacent cells types of the outer retina, interact with each other functionally in numerous ways. Maintenance of permanent retinal adhesion and cyclic phagocytosis of shed photoreceptor outer segment fragments (POS) by RPE cells are two forms of these interactions that are crucial for vision. RPE cells form a polarized monolayer and extend apical microvilli that ensheath photoreceptor outer segments. Outer segments consist of stacked membranous disks containing the phototransduction machinery and are permanently renewed. To maintain constant outer segment length photoreceptors eliminate their most aged tips by daily shedding (Young, 1967), which precedes a burst of phagocytosis by the RPE that efficiently clears POS from the subretinal space and recycles many of their components (Young and Bok, 1969). POS shedding and subsequent phagocytosis by RPE cells are critical for photoreceptor cell function and long term survival. Indeed, complete failure to ingest POS by RPE cells from the Royal College of Surgeons (RCS) rat strain causes debris accumulation and rapid photoreceptor degeneration (Mullen and LaVail, 1976; Edwards and Szamier, 1977). Clearing their daily load of POS renders post-mitotic RPE cells the most active phagocytes in the body.

Synchronized POS clearance is tightly regulated and any delay in completing the shedding or digestion process can cause accumulation of autofluorescent lipofuscin inclusion bodies containing a complex mix of proteins and lipids that likely result from incomplete turnover of POS material (Feeney, 1978). *In vitro* studies have recently shown that lipofuscin components may directly impair RPE function and viability (Finnemann et al., 2002; Schutt et al., 2006). These data suggest that defective digestion of POS by RPE cells may contribute to development or progression of age-related retinal diseases such as age-related macular degeneration.

Outer segment renewal in higher vertebrates is synchronized by circadian rhythms influenced by the daily dark-light cycle (Goldman et al., 1980). Animal studies in rod- or cone-dominant species revealed that rods mainly shed their POS within 2 hours after onset of light and cones shed within 2 hours after dusk (LaVail, 1976; Young, 1977). The increase in the number of phagosomes present in RPE cells at these two time points suggests a peak in phagocytic activity every 12 or 24 hours for RPE cells depending on whether they serve

rods, cones or both. No untimely phagocytosis has been observed so far, suggesting that RPE cells may downregulate their phagocytic activity if not “on duty”.

Retinal adhesion is equally essential for vision as daily POS phagocytosis but must be maintained permanently. Different factors such as intraocular pressure and a net fluid transport from retina to RPE contribute to retinal adhesion. Additionally, receptors expressed at the RPE apical surface are thought to adhere to ligands in the interphotoreceptor matrix (IPM), a complex mix of proteins and proteoglycans filling the subretinal space and ensheathing cone and rod POS (Hageman et al., 1995; Hollyfield et al., 1989; Hollyfield et al., 1999). IPM proteoglycan rearrangement and RPE microvilli collapse are early responses to retinal detachment that, if persistent, result in RPE dedifferentiation and proliferation, POS degeneration and photoreceptor cell death by apoptosis (Cook et al., 1995).

Despite their obvious importance for vision, we still know little about mechanisms that regulate the rhythm of RPE phagocytosis and RPE surface receptors or IPM ligands that mediate retinal adhesion.

2. STUDYING POS PHAGOCYTOSIS BY RPE CELLS *IN VITRO* AND *IN VIVO*

Studies seeking to identify the molecular machinery used by photoreceptors and RPE cells for photoreceptor outer segment renewal greatly benefit from the fact that RPE cells in tissue culture retain their phagocytic activity towards POS. Recording the binding and internalization kinetics of POS by RPE in culture ideally complements the classical microscopic characterization of OS uptake by RPE *in vivo*. First, OS recognition cannot be studied separately from OS internalization *in vivo* because shed OS in the subretinal space are juxtaposed to the RPE surface whether or not they are recognized or bound by RPE receptors. Second, far greater numbers of RPE cells can be evaluated in each sample in *in vitro* assays than in tissue sections. Therefore, small but significant alterations in RPE phagocytic activity may be detected in *in vitro* assays that may be missed in the light and electron microscopy studies of post-mortem tissues. Third, RPE cells *in vitro* can be studied following specific manipulation of their phagocytic mechanism by pharmacological compounds, recombinant proteins, protein overexpression or down-regulation, just to name a few. Gain- and loss-of-function approaches are well suited to unequivocally identify critical components of the RPE phagocytic machinery. Fourth, *in vitro* phagocytic challenge of RPE cells allows one to test directly the phagocytic activity of RPE cells towards POS, while altered photoreceptor shedding or IPM may indirectly alter the phagocytic activity by RPE cells *in vivo*. Importantly, however, RPE cells *in vitro* only provide a phagocytic assay system with relevance to RPE phagocytosis *in vivo* if cells are studied as differentiated, polarized epithelial monolayers that assemble their phagocytic machinery at their apical surface. Finally, while all evidence suggests that the phagocytic activity of polarized RPE cells *in vitro* retains the primary characteristics of the phagocytic activity of RPE cells *in vivo*, this is not the case for the nature of particle contact. In experimental phagocytosis assays, RPE cells must establish firm binding of isolated POS that is stable enough to withstand shear forces during sample processing, including vigorous washing steps. This is in sharp contrast to the contact of apical RPE receptors with shedding/shed POS in the subretinal space, where mechanical stress is absent and a stable binding event per se may not occur. Thus, comparison of *in vivo* and *in vitro* RPE phagocytosis counting fluorescence- or opsin antibody-labeled POS as illustrated in Figure 1 are both required to fully elucidate the phagocytic machinery of the RPE.

3. RPE PHAGOCYTOSIS *IN VITRO* AND *IN VIVO* USES $\alpha\text{v}\beta 5$ INTEGRIN

$\alpha\text{v}\beta 5$ integrin expression at the apical surface of rodent RPE *in vivo* coincides with postnatal establishment of mature interactions between photoreceptors and RPE including the onset of POS renewal (Finnemann et al., 1997). Stable binding of POS to the cell surface of RPE cells in culture is largely dependent on $\alpha\text{v}\beta 5$ integrin receptors (Finnemann et al., 1997). In addition to POS recognition or tethering RPE cells also use $\alpha\text{v}\beta 5$ integrin receptors to activate signaling pathways through focal adhesion kinase that are necessary to activate MerTK (Finnemann, 2003; Nandrot et al., 2004). As $\beta 5$ integrin only dimerizes with αv integrin subunits, $\beta 5$ integrin knockout mice provide the opportunity to study RPE cells that permanently and exclusively lack $\alpha\text{v}\beta 5$ receptors (Huang et al., 2000).

To determine whether $\alpha\text{v}\beta 5$ integrin plays a role in RPE phagocytosis, we used the technology outlined above to quantify phagocytosis of photoreceptor outer segment fragments (POS) by $\beta 5$ -deficient RPE *in vivo* and *in vitro* (Nandrot et al., 2004). Indeed, RPE cells in $\beta 5$ integrin knockout mice *in vivo* retain basal levels of phagocytic activity but lack the characteristic burst of phagocytosis upon early morning rod shedding. Moreover, $\alpha\text{v}\beta 5$ integrin is essential for POS binding *in vitro*, as RPE cells derived from $\beta 5$ integrin knockout mice in primary culture largely fail to bind isolated POS. These data demonstrate that $\alpha\text{v}\beta 5$ integrin has a key function in RPE phagocytosis.

4. STUDYING THE STRENGTH OF RETINAL ADHESION

Integrins can directly mediate adhesive interactions of cells with their extracellular substrate or with neighboring cells. Because of the fragility of isolated neural retinas, *in vitro* assays that study let alone quantify adhesive interactions between photoreceptor outer segments and RPE cells of mammalian origin do not exist. Furthermore, retinal adhesion of intact tissue is known to rapidly decline postmortem in rodent retina. To measure strength of retinal adhesion in retinal tissue immediately post-mortem, we adapted a published protocol to directly compare strength of retinal adhesion from wild-type and knockout mice of different ages as depicted in figure 2a (Endo et al., 1988; Nandrot et al., 2006).

During peeling, the apical domain of RPE cells separates from the basal domain and fractions with the isolated neural retina from wild-type mice if adhesion between the photoreceptors and RPE microvilli is sufficiently strong (Figure 2b). After peeling, both proteins and melanin pigment derived from RPE cells can be analyzed and quantified. Control experiments established that neural retina content of the RPE specific protein RPE65 and of melanin directly correlated with each other and very well represented the number of RPE apical patches attached to the neural retina. Interestingly, both melanin pigment quantification (Figure 2c) and analysis of proteins on western blots (Figure 2d) indicated that retinal adhesion, like retinal phagocytosis, followed a diurnal rhythm with maximum strength of adhesion at ~9.30 AM (3.5 hours after light onset).

5. A ROLE FOR $\alpha\text{v}\beta 5$ INTEGRIN IN RETINAL ADHESION

To determine whether $\alpha\text{v}\beta 5$ integrin receptors at the surface of RPE cells participated in retinal adhesion, we used the neural retina separation technique to quantify strength of retinal adhesion in age- and background-matched $\beta 5$ knockout and wild-type mice (Nandrot et al., 2006). RPE pigment was transferred onto peeled retina from $\beta 5$ knockout mice to a much lower extent, showing a decreased adhesion due to the absence of $\alpha\text{v}\beta 5$ integrin receptors (Figure 2b).

Furthermore, melanin and RPE protein quantification revealed that retinal adhesion was significantly reduced at all times in $\beta 5$ knockout mice (Figure 2c, d). Interestingly the daily

cyclic rhythm of retinal adhesion was attenuated, as the peak was less prominent compared to other time points in these mice. These data demonstrate that $\alpha v\beta 5$ integrin receptors at the surface of RPE cells contribute significantly to retinal adhesion.

6. PERSPECTIVES

Our results show that lack of $\alpha v\beta 5$ integrin receptors eliminates the rhythm of POS phagocytosis in the retina and weakens retinal adhesion. The two functions may be largely independent of each other since both are defective immediately following the establishment of mature RPE-photoreceptor interactions in $\beta 5$ knockout mouse retina. We also found that retinal adhesion and POS phagocytosis, each a crucial function for vision, follow independent diurnal rhythms that peak at different times of day.

$\alpha v\beta 5$ integrin can recognize a number of extracellular ligands such as thrombospondin, vitronectin and MFG-E8. It is therefore an intriguing possibility that apical $\alpha v\beta 5$ receptors of the RPE may respond to two different ligands in the subretinal space to either promote POS phagocytic signaling or to mediate retinal adhesion. We are currently exploring candidates to identify the ligand for each of $\alpha v\beta 5$ integrin's function in the retina *in vivo*.

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Figure 1.

Fluorescence microscopy quantification of POS phagocytosis by RPE cells *in vitro* and *in vivo*. **(a)** Maximal projection of confocal microscopy sections of primary wild-type mouse RPE cells in culture. RPE cells show vigorous uptake of FITC-labeled POS (white) after 1 hour of phagocytic challenge. RPE cell junctions stained with ZO-1 (gray outlines). **(b–d)** Cryosections of 2-month-old wild-type mice eyecups labeled with rhodopsin antibody. **(b)** RPE-photoreceptor outer segment interface close-up showing intact rod outer segments and opsin-positive phagosomes (bright white dots) adjacent to RPE nuclei (gray). **(c–d)** Low magnification view of similar stainings without nuclei illustrates that these images can be used to count phagosome numbers in the RPE. **(c)** One hour before light onset, the RPE cell layer shows few opsin-labeled phagosomes. **(d)** Two hours after light onset, the RPE cell layer shows numerous opsin-labeled phagosomes confirming the daily burst of rod POS phagocytosis by the RPE. Scale bars: 20 μm. Modified from Finnemann and Chang with permission from Humana Press Inc.

Figure 2.

Retinal adhesion is dramatically reduced in $\beta 5$ knockout mice. **(a)** After enucleation and lens/cornea removal, retinas are swiftly peeled off opened eyecups creating shearing forces to assess retinal adhesion. In retinas with normal retinal adhesion, apical cellular domains of RPE largely remain attached to the outer surface of the neural retina. **(b)** Whole-mount of peeled retina, shown outer retina up, demonstrating that $\beta 5$ knockout retina retains significantly less RPE pigment than wild-type retina. **(c)** Quantification of RPE pigment in retina peeled at different times of day. $\beta 5$ knockout retina shows reduced pigment contents and attenuated adhesiveness peak compared to wild-type retina. **(d)** Representative immunoblots of individual peeled retina confirming the melanin pigment results. Modified from Finnemann and Chang and from Nandrot et al. (2006) with permission from Humana Press Inc. and the American Physiological Society.