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Loss of endothelial planar cell polarity and cellular clearance mechanisms in age-related macular degeneration

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Abstract

Apoptosis, autophagosomes, and lysosomes are lacking in the retinal pigment epithelium (RPE) of age-related macular degeneration (AMD) eyes. Necrosis, not apoptosis, appeared to be the prominent type of cell death in RPE, which led to the accumulation of cell debris within and on both sides of Bruch's membrane. The endothelium of the choriocapillaris had an altered planar cell polarity which encompassed the disappearance of fenestrations, the thickening of cytoplasm, and anterior nuclear dislocation. There were no significant differences in RPE and choroidal aberrations between macular and temporal regions. Loss of endothelial polarity could be at the crux of AMD initiation and progression.

Keywords

Age-related macular degeneration (AMD); apoptosis; autophagy; Bruch's membrane; choriocapillaris; endothelial cells; lysosomes; necrosis; planar cell polarity (PCP); retinal pigment epithelium (RPE)

Introduction

Age-related macular degeneration (AMD) is the primary contributor to central irreversible blindness in the elderly population [1]. AMD causes 54% of all blindness in Americans of European ancestry and 5% of global blindness [2]. Although many contributing factors to AMD development are well known and its late-stage phenotypes are well-characterized, AMD's etiology, especially the early events of its initiation and progression, remains elusive [3]. Despite the extensive investigations of its immunological, genomic, and clinical aspects, AMD is still poorly studied ultrastructurally [4].

This study aims to interpret the ultrastructural aberrations in retinal pigment epithelium (RPE), Bruch's membrane (BM), and the choriocapillaris (CC) plexus of AMD in order to identify the contributing factors to AMD's etiology since very few published ultrastructural reports on AMD have identified pathological abnormalities in structures [5,6]. Our current report focuses on three aspects of AMD: (1) the type of cell death and clearance processes

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Declaration of interest

The authors declare no conflict of interest.

within the RPE, (2) the state of the CC and its endothelial cell polarity, and (3) the comparison between the macular and temporal regions of these two aspects.

The endothelial layer has a polarity that is achieved by differentiating into two zones: the inner zone abutting BM has a very thin cytoplasm that is punctuated by numerous fenestrae and the choroidal zone with a thick cytoplasmic margin where the nuclei are located [7]. By comparison, the retinal capillaries have no polarity [8–10].

Endothelial cell differentiation includes spatial orientation and polarization [11,12]. Within the CC, endothelial polarity includes planar cell polarity (PCP), which is usually described as the differentiation of an endothelial sheet of cells into a perpendicular orientation to the apical–basal axis and entails the asymmetric localization of protein complexes within individual cells [13]. The PCP pathway (the noncanonical Wnt pathway [β -catenin-independent]) is evolutionarily conserved from *Drosophila* to mammals and controls several cellular differentiation processes that include epithelial cell polarization, cell migration, and mitotic spindle orientation [14,15]. In addition, PCP factors have been implicated in some diseases, and some have been linked to the genetic syndromes of ciliary dysfunction such as polycystic kidney disease [12]. Although the PCP field has investigated a number of tissues and diseases of different species, it has not yet tackled the issue in the CC.

While the homeostatic status of the CC is influenced by the RPE, changes in the choroid can have an impact on the RPE; for example, choroidal ischemia and congestion may lead to lesions and RPE–retina dysfunction [16,17]. Aberrations within one of the components of the RPE–Bruch’s–choroid complex compromise the homeostasis of the RPE and choroid [16,18,19]. Several studies have pointed out the reduction in CC flow in AMD and attributed it to the buildup of deposits within BM [17,20–22], a condition we also have observed in the AMD specimens of this study.

Thus far, the subject of endothelial polarity loss (EPL) in AMD has not been fully investigated [5,7,23]. It is also unknown if loss of endothelial fenestration is associated with EPL. Furthermore, there is no citation in the literature on whether aging is a factor in EPL. This void provides us with the opportunity to explore the causes and consequences of EPL.

Our survey identifies ultrastructural aberrations in RPE, BM, and CC of AMD. These include the lack of apoptosis, autophagy, and lysosomes in RPE, the accumulation of drusen and cellular debris, as well as the loss of polarity in endothelial cells of the CC. We also report on whether changes within the macula differ from those in the peripheral fundus.

Materials and methods

An ultrastructural examination was performed on the choroid as well as RPE macular and peripheral regions of seven AMD donors (five eyes from the National Disease Research Interchange, Philadelphia, PA, and the other two from in-house autopsies). All the donors were over 80 years with dry AMD. The eyes were formalin fixed, and the macular and temporal regions were dissected out separately. The specimens were prepared for transmission electron microscopy (TEM) according to Ogilvy et al. [24]; briefly, tissues were embedded in epoxy resin, sectioned at 90 nm thickness, stained with uranyl acetate and

lead citrate, then viewed with JEOL JM-1010 TEM. The diameters of the CC vessels adjacent to BM were measured in the macular and temporal regions.

Results

There were no significant differences in the RPE and choroidal aberrations between the macular and temporal regions (Figure 1A, B). Normal and necrotic RPE cells appeared without lysosomes or autophagosomes, and none of the RPE cells were exhibiting any signs of apoptosis (Figure 2B–D). In some specimens, the RPE layer had doubled by producing a new layer of RPE cells on top of the necrotic cells; however, basal infoldings were absent (Figure 2B–D). Drusen or cellular debris of necrotic RPE cells, present below the RPE layer, were comprised of lipid droplets, lipofuscin, chromatin vesicles, degenerate melanosomes, degenerate mitochondria, aqueous vesicles, and ultrafine cellular fragments (Figure 2B–D). Chromatin vesicles are spheroidal structures that are made of extranuclear DNA (enDNA) chromatin strands that leak out of the nucleus; a full description with high-resolution images of chromatin vesicles can be found in Ogilvy et al. [24]. BM was altered due to collagen degeneration and loss of elastin. This damage allowed drusen contents and cellular debris of necrotic RPE cells to pass through BM from the RPE side into the choroidal side (Figure 2B–D).

Endothelial cells of the CC showed either a significant decrease in the number of fenestrations or a total loss of fenestrations (Figure 3A–D). The cytoplasm was anteriorly hypertrophic or completely absent (Figure 3B, C). Endothelial nuclei were mostly hypertrophic, and the majority were anteriorly displaced toward BM, as opposed to the usual posterior or lateral positions (i.e., loss of normal polarity) (Figure 3B, D).

Pericytes were degenerate and the basement membranes were scant (Figure 3C, D).

The average diameter of capillaries was within the normal range in both the macula and temporal regions (6.1–7.8 μm).

Discussion

Our survey of AMD specimens revealed a pattern of ultrastructural aberrations, some of which have not been previously described in detail or were suggested only by molecular studies. The RPE aberrations indicate dysfunctional cellular clearance processes, or their absence, including apoptosis, autophagy, and lysosomes. The prevalent type of cell death contributing to the various AMD phenotypes is a point of controversy. Apoptosis has been the preferred explanation of RPE degeneration for quite a while; however, recent molecular and ultrastructural studies point to pyroptosis and necroptosis as the principal mechanisms of cell death [3]. Apoptosis, autophagy, and lysosomes are cellular processes that are deployed to maintain a healthy tissue and reduce the potential of inflammation and pathogenesis [3,25,26]. Therefore, the inability of the cell to carry out one or more of these processes will create abnormal conditions within the cell and cause degenerative diseases such as AMD. Although the molecular aspects of these processes have been studied in AMD, they have not been adequately studied ultrastructurally in sizable cohorts.

Recent studies on AMD, including our own, have pointed out the lack of apoptosis within the RPE layer and the propensity of its cells to undergo necrosis [3,27]. Necrosis differs from apoptosis in its disorderly breakdown of the cell and the release of its partially digested contents within the tissue. Apoptosis, however, turns the cell into a digestive sac (the apoptotic body) where all cellular contents breakdown and later the apoptotic body is engulfed by a macrophage [3]. Lysosomal dysfunction, mostly due to increased alkalization within lysosomes, has been suggested as a probable inducer of an AMD phenotype in mouse and rat models as well as cultured RPE cells [28,29]. However, our study suggests that lysosomes are absent within the pathological RPE. Mitter et al. concluded that autophagy is dysfunctional in AMD by showing that autophagy proteins, autophagosomes, and autophagy flux were significantly reduced in AMD of human tissue and two animal models of AMD [30]. Others have shown that dysfunctional autophagy and heterophagy could cause AMD [25].

Chromatin vesicles were visible in the drusen. We have previously described these vesicle formation in a mouse model of AMD, defined them as a form of enDNA, and showed similar structures in human specimens of AMD [3,24]. Others have speculated on the presence of double-stranded RNA in the drusen, but did not confirm it [31]. Cytosolic leakage of nucleic acids can trigger inflammatory reaction when their detectors are activated; for example, enDNA activates cGAS protein to produce an inflammatory immune response by interferon- β (INF- β) through the cGAS–cGAMP signaling pathway [32]. Chromatin leakage and enDNA vesicle formations in drusen are most likely a later event in AMD, with inflammatory consequences that will maintain the downhill effect of hypoxia (due to the mitochondrial destruction by cytokines) and establish chronic inflammation [3].

Our data suggest that RPE cells are forced into necrosis, leading to the accumulation of cellular debris on top of and within BM, then passed onto the choroidal side of BM. The prevalence of necrosis as opposed to apoptosis lends itself to the hypothesis of the CC aberrations preceding RPE degeneration [20]. Apoptosis is an energy-expensive process; therefore, a nutrient- and energy-deprived cell would not be able to effectively carry out and complete the apoptotic process [33].

The CC plexus is a highly polarized vascular structure characterized by an attenuated and fenestrated endothelium at the inner side abutting BM [7]. It provides significant perfusion to the RPE; this is facilitated at the fenestration zone where the endothelial cell membrane is composed of a single layer; thus there is no cytoplasmic barrier between the vessel lumen and basement membrane of the capillary [8,10,34]. Normal innermost vessels of the CC plexus have a particular endothelial organization termed PCP. PCP is characterized by having fenestrations and scant cytoplasmic density on the inner side of the vessels' endothelium with nuclei located at the outer or lateral sides of the vessels [10]. Our current survey has shown that PCP is disrupted in the AMD eyes where fenestrations were mostly missing, thickened, or lacking inner cytoplasm, and nuclei relocated to the inner side. Loss of PCP results in limited perfusion at the affected site, which could contribute to RPE hypoxia, the consequent necrosis, and drusen formation. This is especially significant if PCP loss is an early event in AMD etiology. PCP within the CC is established by the interaction of two cellular pathways: Wnt's noncanonical PCP pathway and vascular endothelial growth

factor-targeted (VEGF-targeted) pathway [35,36]. Only in choroidal neovascular AMD serum VEGF levels are higher; however, this could be a later event in AMD [37]. Thus far, the mechanisms by which these two pathways contribute to PCP loss and AMD initiation remain unresolved.

Whether AMD development is caused by RPE problems or the CC alterations remains a major query and awaits further investigations [20,38]. For example, Korte et al. found that the destruction of the RPE preceded the degeneration of the CC and the latter only degenerated in places where the RPE was lost [38]; however, the authors did not report on the loss of PCP. On the other hand, the ultrastructural aberrations in RPE cells reported here indicated dysfunctional cellular clearance processes such as apoptosis, autophagy, and lysosomes, which are all energy sensitive and can become dysfunctional due to a lack of perfusion [33]. Thus, if the loss of PCP is an early event in AMD, it would lend support to a defective CC as the precursor to RPE insufficiency.

The formation of fenestrations in the endothelial cells appears to be a later step in the vessel's PCP ontogenesis, but its details have not been resolved. Although several studies attribute fenestration development in the CC plexus and the glomerular endothelial cells to VEGF regulation [39–41], none discuss the contributions of the noncanonical Wnt signaling pathway. Endothelial cells that do not form in a VEGF gradient have been shown to exhibit loss of cell polarity [13]; however, noncanonical Wnt5a can act in a similar way as a pro-angiogenic factor promoting endothelial cell proliferation, migration, survival, and network formation as well as vessel stabilization [42].

The most significant ultrastructural aberrations within the CC of AMD eyes were the endothelial abnormalities such as the loss of polarity of the endothelial cells. Nuclei of normal endothelial cells are located posteriorly at the scleral side of the capillary, not anteriorly at the side of BM, as seen in our study. Cellular proliferation, polarity, and apoptosis have been found to be regulated by the Frizzled Wnt signaling pathway [36]. A phenotype of disrupted cell polarity has been observed in deviant downstream signaling through small GTPases (i.e., Rac1, Cdc42, and RhoA) of the Wnt pathway [13].

Aging individuals develop atherosclerosis in their blood vessels. Atherosclerosis can cause significant perfusion reduction due to plaque formation and constriction of vessels' lumina. Additionally, atherosclerosis causes the buildup of free radicals that can cause damage to the vascular wall [43] and restricts the oxygen supply needed to meet the requirements of energy-expensive pathways; this encompasses a number of GTPases that are required for proper functioning and maintenance of cellular polarity. With the loss of polarity and destruction of fenestrations, the RPE would lack sufficient nutrients for proper function, which could lead to substantial insufficiency.

In addition to the above findings, both the macular and peripheral regions in the eye shared the same abnormalities in their RPE and CC. There were no significant differences between the macular and peripheral regions, which indicates a systemic rather than local aberration.

In conclusion, the widespread loss of endothelial polarity in the CC supports the hypothesis of a CC dysfunction resulting in a RPE insufficiency. This is reinforced by the observation

that vascular damage appears to be a systemic condition within the outer region of the CC plexus. Further study is necessary to provide better support for this claim; however, when examined independently at the ultrastructural level, it is clear that there are major vascular aberrations and perfusion problems which provide a plausible explanation for the downstream disruption of RPE.

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Figure 1. Light microscopy of cross-sections through the RPE, Bruch's membrane (BM), and choriocapillaris (CC) showing drusen, cell debris accumulation below the RPE, and the distorted RPE layers. (A) Macular region. (B) Peripheral region.

Figure 2.

Drusen and cell debris accumulation below and above RPE, within Bruch's membrane (BM), and above choriocapillaris (CC). (A) Normal RPE, BM, and fenestrations of CC; inset shows high magnification of normal fenestrations (arrows). Both images are from wild-type C57BL/6J mouse. (B) Drusen formation from a necrotic RPE cell that encompasses lipofuscin, degenerate melanosomes, degenerate mitochondria, vesicles of various sizes, chromatin vesicles, and other cellular debris. Bruch's membrane is degenerate and packed with cellular debris. (C and D) Necrotic RPE cells showing degenerate cytoplasm and nuclei on the top of deteriorated Bruch's membrane.

Figure 3. Endothelium loss of polarity (EPL) in three AMD patients. Sample EM images of choriocapillaris (CC) showing loss of endothelial fenestration (A–D), thickening of cytoplasm (arrows, A–D), and endothelial nucleus (EN) hypertrophy (B) and its relocation to the side of Bruch's membrane (C & D [marked by asterisk in D]).