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Serum starvation of ARPE-19 changes the cellular distribution of cholesterol and Fibulin3 in patterns reminiscent of age-related macular degeneration

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ABSTRACT

Retinal pigment epithelium (RPE) has been implicated as key source of cholesterol-rich deposits at Bruch's membrane (BrM) and in drusen in aging human eye. We have shown that serum-deprivation of confluent RPE cells is associated with upregulation of cholesterol synthesis and accumulation of unesterified cholesterol (UC). Here we investigate the cellular processes involved in this response. We compared the distribution and localization of UC and esterified cholesterol (EC); the age-related macular degeneration (AMD) associated EFEMP1/Fibulin3 (Fib3); and levels of acyl-coenzyme A (CoA): cholesterol acyltransferases (ACAT) ACAT1, ACAT2 and Apolipoprotein B (ApoB) in ARPE-19 cells cultured in serum-supplemented and serum-free media. The results were compared with distributions of these lipids and proteins in human donor eyes with AMD. Serum deprivation of ARPE-19 was associated with increased formation of FM dye-positive membrane vesicles, many of which co-labeled for UC. Additionally, UC colocalized with Fib3 in distinct granules. By day 5, serum-deprived cells grown on transwells secreted Fib3 basally into the matrix. While mRNA and protein levels of ACTA1 were constant over several days of serum-deprivation, ACAT2 levels increased significantly after serum-deprivation, suggesting increased formation of EC. The lower levels of intracellular EC observed under serum-deprivation were associated with increased formation and secretion of ApoB. The responses to serum-deprivation in RPE-derived cells: accumulation and secretion of lipids, lipoproteins, and Fib3 are very similar to patterns seen in human donor eyes with AMD and suggest that this model mimics processes relevant to disease progression.

1. Introduction

Retinal pigment epithelium (RPE) cells are crucial for retinal homeostasis and visual function [1]. The key homeostatic functions of RPE include phagocytosis of shed photoreceptor outer segments; formation of the blood retinal barrier; transportation of nutrients, water, and ions to the retina and removing metabolic waste from the retina to the choroidal vasculature [2]. The role of RPE in the visual cycle includes absorption of light, protection against photo-oxidation and recycling of all-trans-retinol to 11-cis retinal to maintain the visual cycle [3].

During aging, RPE cells undergo numerous structural changes that typically progress gradually and at varying rates among individuals [4]. These include increased density of residual bodies or undigested outer segments, and an accumulation of lipofuscin pigment and basal deposits on or within BrM [5]. The aging process also results in the accumulation of lipids, formation of druse between the basal lamina of the RPE and inner collagenous layer of BrM, microvilli atrophy, disruption of the basal in-folding, and thickening of the BrM [6,7].

Studies investigating the accumulation and composition of lipids in BrM/choroid implicate both plasma and local cells as the source of the lipids [6,8,9]. RPE especially has been implicated as a secretor of esterified cholesterol (EC)-rich ApoB to BrM [10]. Initially it was hypothesized that ApoB lipoprotein from RPE removes fatty acids released by lysosomal phospholipases after phagocytosis of shed outer segments [11], however, BrM lipoproteins and drusen are more highly enriched in EC and unesterified cholesterol (UC) than outer segment membranes [12]. Thus, the source of lipids found in RPE lipoproteins is not yet clear.

Confluent human-derived ARPE-19 cells express scavenger receptor

Abbreviations: RPE, retinal pigment epithelium; UC, unesterified cholesterol; EC, esterified cholesterol; Fib3, EFEMP1/Fibulin3; AMD, age-related macular degeneration; ApoB, Apolipoprotein B; BrM, Bruch's membrane; HDL, high-density lipoprotein; FCS, fetal calf serum; SFM, serum free medium; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; PFA, paraformaldehyde; CFH, Complement Factor H; ER, endoplasmic reticulum; BLinD, basal linear deposits; ACAT, Acyl-coenzyme A (CoA): cholesterol acyltransferases; SRB-I, scavenger receptor B-I

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B-I (SRB-I) for high-density lipoprotein (HDL) [13]. Furthermore, we have shown that ARPE-19 cells also express functional receptors for LDL (LDLR), and respond to serum deprivation by upregulating expression of genes in the lipid and cholesterol pathways as well as accumulating intracellular UC [14]. A similar transcriptional response is also seen in primary human fetal RPE. Serum-deprived ARPE-19 cells also show increased secretion of the extracellular matrix protein Fib3 which is deposited basally in AMD [15,16]. Accumulating defects in BrM and dysfunction of the choriocapillaris may obstruct nutrient transport and signaling to the RPE and retina. The response to serum deprivation by RPE cells could contribute to upregulated cholesterol synthesis and eventually to the secretion and accumulation of cholesterol and lipids associated with the progression of AMD.

Here, we examine the behavior of ARPE-19 cells under serum deprivation. Our results show that under serum deprived conditions UC is synthesized by ARPE-19 in the endoplasmic reticulum (ER) and accumulates intracellularly, whereas EC lipid droplet accumulation is lower than in cells in serum. The effects of serum deprivation on ARPE-19 cells include increased formation of FM dye-positive membrane vesicles, and increased basal secretion of Fib3. Both the membrane vesicles and Fib3 co-localize with UC. Serum deprivation also increased the expression of ACAT2 and secretion of ApoB lipoprotein from ARPE-19 cells. ACAT2 promotes cholesterol esterification and stimulates cholesteryl ester secretion in ApoB-containing lipoproteins [17], suggesting that the lower levels of intracellular EC under serum-deprivation may be associated with increased formation and secretion of ApoB lipoproteins.

2. Material and methods

2.1. In vitro RPE cell culture

ARPE-19 cells were purchased from ATCC (ATCC, Manassas, VA, CRL-2302; passage numbers 5–15), were cultured in complete Dulbecco's Modified Eagle Medium F12 (Gibco Life Technology, Gaithersburg MD; DMEM F12) medium containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (100 unit penicillin/100 μg streptomycin per ml), (Invitrogen, San Diego, California, USA). Cells were grown in an incubator at 37 °C with 5% CO2 using T25, T75 tissue culture flasks, tissue culture plates or collagen-coated transwell inserts (Corning Primaria plastic culture ware, Thermo Fisher Scientific, Waltham, MA). ARPE-19 cells were authenticated using short tandem repeat (STR) analysis by the cell line authentication service (ATCC). The cell line used has been published previously and the cells were free from contamination. ARPE-19 cells were validated for the expression of the RPE65 and RLBP1 marker genes [18] with PCR of cDNA using the RPE65-specific primers 5′-CCA GAT GCC TTG GAA GAA GA-3′; 5′-CTT GGC ATT CAG AAT CAG GAG-3′ (99 bp amplicon) and the RLBP-specific primers 5′-AGA TCT CAG GAA GAT GGT GGA C-3′; 5′-TGG ATG AAG TGG ATG GCT TT-3′ (72 bp amplicon) [14].

2.2. Transwell culture system for ARPE-19 cells

Permeable supports enable cells to grow in a polarized state under more natural conditions and results in cells that are morphologically and functionally better representative of in vivo cells. In this model, cells were grown on collagen-coated 24 mm transwell inserts (Corning Primaria plastic culture ware, Thermo Fisher Scientific, Waltham, MA) for 4 weeks before serum starvation experiments. The permeable membranes generate two compartments, the apical (upper compartment) domain corresponds to the retinal facing side of the RPE monolayer and the basolateral (lower compartment) domain corresponds to the choroidal/BrM facing side of the RPE monolayer.

For live cell imaging, the membrane immersed in culture media was dissected and placed with the cells facing downward on in 100 μL of media in a petri-dish and imaged using a Zeiss LSM 880 microscope with Airyscan (Zeiss, USA). For membrane sections, the membranes with cells fixed in 4% PFA for 15 min were glued, cells facing upward, onto a 4 mm thick 5% agarose gel. The blocks with attached membrane were cut into 100 μm thick sections using a Leica_VT1000S_Vibratome.

Table 1
Donor eyes described in this paper.

NDRI	Genotype	Description	Age/sex
#0068299	H/Y	Normal	76/M
#0068536	H/H	Dry AMD	78/F
#0068280	Y/Y	Wet AMD	85/M

2.3. Serum deprivation

After reaching confluence in serum supplemented media culture medium was removed and the cells were washed once with serum free medium (SFM) before re-incubating in SFM, DMEMF12 with 1% penicillin/streptomycin (100 units penicillin/100 μg streptomycin per ml). Day 0 in all experiments denotes cells that remained in complete culture medium (10% serum) throughout the experiment. Days 1, 3, 5 and 9 represent cells in SFM.

2.4. Western blot

ARPE-19 cells washed with 1X phosphate-buffered saline (PBS; KD Medical, Columbia MD: catalog# RGF-3190) were either lysed in RIPA buffer with protease inhibitors (Thermo Fisher Scientific) or culture supernatants were collected. Protein concentrations were measured using a BCA assay kit (Thermo Fisher Scientific). 20 μg of total protein was loaded onto 10% SDS-PAGE gel. Gels were run at 80 V for 30 min followed by 150 V for 60 min. Proteins were transferred to Immobilon-FL polyvinylidene difluoride (PVDF) membrane at 350 mA for 50 min. Secreted protein blots were transferred to 5 ml of Ponceau S staining solution for 5 min, and washed thoroughly with 5% acetic acid solution (v/v) before continuing with blocking. All blots were blocked with 5% bovine serum albumin (BSA) in tris-buffered saline with Tween-20 (TBS/T) for 1 h at room temperature then rinsed once in TBS/T. Next the blots were incubated with primary antibodies diluted 1:1000 with TBS/T overnight at 4 °C. Rabbit polyclonal anti-ACAT1 (Abcam), rabbit polyclonal anti-ACAT2 (Abcam) and rabbit anti- EFEMP-1 (Century Biochemicals) were used as primary antibodies. After thorough washes, the membranes were incubated with HRP-conjugated secondary antibodies diluted 1:10000 for 2 h in the dark at room temperature. Finally, the membranes were washed in TBS/T 3 times before scanning using LumiGold ECL Western Blotting Detection Kit (VerII; Signagen Laboratories, Ijamsville, MD). The blots shown are representative of at least three biological repeats of each experiment. The β-actin level or Ponceau S stained image was used to normalize the signal from other proteins. The Western blot signals were quantitated using ImageJ software (version 1.45; National Institutes of Health, Bethesda, MD).

2.5. Immunofluorescent labelling and staining of cells

ARPE-19 cells cultured on cover slips, chambers or transwell inserts were washed with cold PBS and fixed with 2% paraformaldehyde (PFA) for 10 min, followed by permeabilization with 0.1% Triton-X for 5 min. The samples were blocked with 5% BSA for 30 min at room temperature. Cells were incubated with EFEMP-1(Fib3) (Century Biochemicals) or rabbit polyclonal ZO-1 (Abcam) primary antibody diluted 1:100 for 4 h. After washing with PBS, samples were incubated with anti-rabbit 488 or 568 (Thermo Fisher Scientific) secondary antibody diluted 1:100 with PBS and counter stained with DAPI diluted 1:500 in the dark for 1 h. FM dye (Thermo Fisher Scientific) was added to live cells for 1 min at room temperature, Hoechst 33342 for 30 min at 37 °C and CellLight