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Expression of reverse cholesterol transport proteins ATP-binding cassette A1 (ABCA1) and scavenger receptor BI (SR-BI) in the retina and retinal pigment epithelium

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Abstract

Aims—Excessive lipid accumulation in Bruch’s membrane (BrM) is a hallmark of ageing, the major risk factor for age-related macular degeneration (AMD). Retinal pigment epithelial (RPE) cells may utilise reverse cholesterol transport (RCT) activity to move lipid into BrM, mediated through ATP-binding cassette A1 (ABCA1) and scavenger receptor BI (SR-BI).

Methods—ABCA1 expression was assessed by reverse transcription polymerase chain reaction (RT-PCR) and western blotting of human RPE cell extracts. Lipid transport assays were performed using radiolabelled photoreceptor outer segments (POS). ABCA1 and SR-BI expression was examined in normal mouse eyes by immunofluorescence staining. BrMs of ABCA1 and SR-BI heterozygous mice were examined microscopically.

Results—Human RPE cells expressed ABCA1 mRNA and protein. The ABCA1 and SR-BI inhibitor glyburide (also known as glibenclamide) abolished basal transport of POS-derived lipids in RPE cells in the presence of high-density lipoprotein. Mouse retina and RPE expressed ABCA1 and SR-BI. SR-BI was highly expressed in RPE. BrMs were significantly thickened in SR-BI heterozygous mice, but not in ABCA1 heterozygous mice.

Conclusion—RPE cells express ABCA1 and SR-BI. This implies a significant role for SR-BI and ABCA1 in lipid transport and RCT in the retina and RPE.

Retinal pigment epithelial (RPE) cells play a critical role in retinal lipid trafficking through phagocytosis of photoreceptor outer segments (POS) and lysosomal degradation of POS lipids. While POS phagocytosis by RPE has been studied extensively, little is known about the mechanisms of lipid and cholesterol efflux from RPE cells. Because development of age-related macular degeneration (AMD) is associated with lipid and cholesterol deposition in RPE and BrM, understanding the molecular mechanisms of lipid and cholesterol efflux from the RPE may be critical to understanding the pathogenesis of this condition.^{1,2}

Due to their potential athero-protective effects, mechanisms of lipid efflux have been investigated widely in non-ocular tissues. There are multiple mechanisms for cholesterol

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efflux from cells, including passive diffusion, oxysterol production, apolipoprotein (Apo) E secretion and reverse cholesterol transport (RCT). Efflux to a lipoprotein acceptor is the first step in RCT by which cellular phospholipids and non-esterified cholesterol are effluxed to high-density lipoprotein (HDL) for transport to the liver. In an early step of the RCT pathway, ATP-binding cassette transporter 1 (ABCA1) or scavenger receptor BI (SR-BI) effluxes phospholipids and non-esterified cholesterol from the cell to ApoA-I or lipid-poor HDL at the cell membrane.^{3,4} HDL-bound lipid and cholesterol are then transported from peripheral tissues to the liver for hepatic uptake and biliary secretion.

A homozygous recessive mutation in the ABCA1 gene results in Tangier disease, a rare condition causing premature atherosclerosis, accumulation of lipid and cholesterol in the reticuloendothelial system and cornea, and low levels of plasma HDL.^{5, 6} Retinal abnormalities in Tangier disease have not been reported. Conversely, over-expression of wild-type ABCA1 increases cellular lipid and cholesterol efflux and levels of plasma HDL.⁷ Mice heterozygous for null mutations in SR-BI express about half as much SR-BI protein as wild-type mice and develop elevated levels of plasma cholesterol.⁸

We previously demonstrated that human RPE cells express SR-BI.⁹ Herein, we report that human RPE cells express ABCA1. We also demonstrate that inhibition of ABCA1 and SR-BI activity by glyburide (glibenclamide) abolishes HDL-stimulated basal efflux of photoreceptor-derived lipids in cultured human RPE cells. Finally, we demonstrate abnormalities in BrM morphology in mice heterozygous for a null mutation in SR-BI. Because progressive accumulation of lipids in RPE and BrM is the hallmark histopathological finding in early age-related macular degeneration (AMD), regulation of RCT may play a role in the pathogenesis of this common cause of visual loss.

METHODS

Cell culture

Primary cultures of normal adult or fetal human RPE cells were prepared as described.^{10, 11} Cells from passages four to ten were used. RPE cells were grown on laminin-coated tissue culture plates in DMEM H21 containing 5–10% fetal bovine serum, 2 mmol/l glutamine, 2 ng/ml gentamycin, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml fungizone, 1 ng/ml basic fibroblastic growth factor and 1 ng/ml epidermal growth factor. No differences in cell morphology or protein expression were observed in different cultures. RPE cells were grown at confluence for at least 7 days prior to undergoing the experimental treatments described below.

RT-PCR

RT-PCR was carried out on 1 µg of cDNA. The RT-PCR products were resolved by electrophoresis on 1.4% agarose gels. The RT-PCR primer sequences used are followed by the predicted ABCA1 RT-PCR product size. ABCA1 forward: 59-AAC-AGT-TTG-TGG-CCC-TTT-TC; ABCA1 reverse: 5'-AGT-TCC-AGG-CTG-GGG-TAC-TT, 164 bp product spanning exons 27 and 28. PCR was conducted for 20–30 cycles at 55°C in buffer containing 2.0–5.0 mmol/l MgCl₂. DNA sequencing confirmed the RT-PCR product of the predicted size for ABCA1.

Western blotting

RPE cell proteins were extracted in radioimmunoprecipitation assay (RIPA) buffer (150 mmol/l NaCl, 1% Igepal (Sigma-Aldrich, St Louis, Missouri, USA), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate polyacrylamide (SDS), 50 mmol/l Tris, pH 8.0, 1 mmol/l phenylmethanesulphonyl fluoride (PMSF)). Cell protein (60 mg) was subjected

to SDS–polyacrylamide gel electrophoresis followed by electrophoretic transfer to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, Livingston, New Jersey, USA). Membranes were blocked with 5% dried milk, and probed with anti-ABCA1 antiserum (Novus Biologicals, Littleton, Colorado, USA) diluted 1:1000. After incubation with an anti-rabbit secondary antibody, ABCA1 bands were detected using the ECL chemiluminescence reagent (Amersham Biosciences) followed by exposure to x-ray film.

Cell immunohistochemistry

Cells were fixed in 2.5% paraformaldehyde for 15 min, then stained with antiserum to human ABCA1 (Abcam Inc., Cambridge, Massachusetts, USA). Briefly, slides were incubated sequentially in Biotin Blocking Solution and Avidin D Blocking Solution (Vector Laboratories, Burlingame, California, USA) according to the manufacturer's instructions. The slides then were washed three times with 10 mmol/l sodium phosphate pH 7.8, 150 mmol/l NaCl (buffer A), incubated in rabbit antiserum to human ABCA1 (1:400 dilution) for 30 min at room temperature and washed three times with buffer A. Slides were incubated for 30 min with biotinylated goat anti rabbit antibody (5 µg/ml) (Vector Laboratories) and then washed three times with 100 mmol/l sodium bicarbonate pH 8.5, 150 mmol/l NaCl (buffer B) and incubated for 30 min with fluorescein isothiocyanate (FITC)–avidin (10 µg/ml) (Vector Laboratories), washed three times with buffer B and cover-slipped with Vectashield mounting medium. As controls, immunostaining was performed with primary antibody omitted. Cells were visualised by wide-field epifluorescence microscopy.

Lipid efflux assay

Basal lipid transport was assayed as previously described with a few modifications.¹⁰ Briefly, human fetal RPE cells were grown at confluence on laminin-coated 12-well Transwell® plates (coming Life Sciences, Lowell, Massachusetts, USA). Only cells with transepithelial resistances in excess of 50 Ω/cm² were used. POS were radiolabelled using [¹⁴C]stearic acid. The apical chambers received 50 µg/well [¹⁴C]stearic acid (359,000 cpm) in Chee's replacement medium.¹¹ Basal chambers contained Chee's replacement medium ± 100 µg/ml HDL, and ± 250 µmol/l glyburide, an inhibitor of ABCA1 and SR-BI. After 36 h basal media were collected and subjected to liquid scintillation counting.

Animals

For the SR-BI studies, 8-month-old mice heterozygous for a null mutation that prevents expression of SR-BI (B6;129S2-*Scarb1*^{tm1Kri}/J; Jackson Labs, Bar Harbor, Maine, USA) were evaluated.¹² For the ABCA1 studies, 8-month-old mice heterozygous for a null mutation in ABCA1 (DBA/1-*Abca1*^{tm1Jdm}/J; Jackson Labs) were used.¹² In each of these strains heterozygotes have been shown to express about 50% as much protein as wild-type mice.^{8, 12} Age-matched DBA/1 and C57BL/6J mice were used as controls for the ABCA1 and SR-BI heterozygotes, respectively.

Mouse tissue immunocytochemistry

Mice were killed by overdose of carbon dioxide. Eyes were immersion-fixed in 4% paraformaldehyde for 2 h at room temperature, and the cornea and lens were removed. Eyecups were cryoprotected with 30% sucrose in 0.1 mol/l phosphate buffer overnight, then frozen embedded in optimum cutting temperature medium. Cryostat sections (10 µm) were labelled with rabbit anti-human SR-BI10 or ABCA1 (Abcam Inc.) antibody diluted 1:200 at 4°C overnight. Slides were incubated for 1 h at room temperature with Alexa Fluor® 488 highly cross-adsorbed goat anti-rabbit heavy plus light chain IgG (cat. no. A-11034; Molecular Probes, Eugene, Oregon, USA) diluted 1:500. Blocking reagent consisting of 10% normal goat serum (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and

0.1% Triton X-100 (Sigma-Aldrich, St Louis, Missouri, USA) in phosphate buffered saline (PBS) was applied prior to antibody incubations. As controls, immunostaining was performed with primary antibody omitted. In the case of SR-BI, antigen blocking (pre-incubation with excessive SR-BI immunising peptide with primary antibody) was also performed.

Mouse tissue electron microscopy

Mice were killed by overdose of carbon dioxide and the 12 o'clock positions of the eyes were marked. The eyes were fixed by immersion in 2.5% glutaraldehyde in phosphate buffer overnight, bisected along the vertical meridian and processed for electron microscopy by the osmium–tannic acid phenylenediamine (OTAP) method to preserve neutral lipids.¹³ The posterior region of the retina approximately 1 mm from the optic nerve in the inferior hemisphere of the eyes was photographed by light microscopy. Regions containing the RPE and BrM were photographed by electron microscopy (19,000× initial magnification). BrM thickness was measured on at least 50 points per eye (ten micrographs per eye) at 1 µm intervals (arrows in fig 4A and D). The results were averaged per eye, and average BrM thickness per eye was compared using one eye from at least three mice from each group.

Retinal function assessment

Scotopic and photopic full-field electroretinograms (ERG) were recorded and measured as previously described¹⁴ after dark adaptation overnight in both eyes of three ABCA1^{+/-} mice and three controls, and ten SR-BI^{+/-} adult mice and 13 controls. Mean amplitudes and implicit times were compared with controls. To measure dark adaptation, or recovery of photo-receptor function after light exposure, mice were exposed to a photobleach of 29 cd/m² for 5 min, then scotopic a-wave amplitudes were recorded at 5 min intervals in response to a flash of 0.4 log₁₀ cd/m² over a recovery period of 60 min. For measures of functional recovery, a-wave amplitudes were measured at 10 ms after the stimulus, prior to intrusion of post-receptor responses, and were normalised by dividing by the pre-bleach a-wave amplitude.

RESULTS

We first determined whether primary cultures of RPE cells express mRNA and protein for ABCA1. As shown in fig 1, ABCA1 mRNA was detected by RT-PCR (fig 1A, panel 1), and the 250 kDa ABCA1 protein was detected by western blotting (fig. 1A, panel 2). The 500 kDa protein band represents ABCA1 dimers. As shown in fig 1B, ABCA1 was also detected in RPE cells by immunofluorescence staining. ABCA1 staining was observed throughout the cells.

We next determined if ABCA1 and/or SR-BI functions are implicated in RCT from the basal surface of cultured RPE cells. Basal lipid transport experiments were conducted using the ABCA1 and SR-BI inhibitor glyburide (fig 2). Inclusion of HDL in the basal medium increased lipid efflux by about 1.5-fold compared with control (p=0.0129). Inclusion of glyburide had no effect on lipid efflux in the absence of HDL. However, when glyburide was included with HDL, HDL stimulation of basal lipid efflux was abolished (p= 0.0319).

Having demonstrated that cultured human RPE cells express ABCA1 (above) and SR-BI (previously⁹), we assessed the effect of mutation of the genes that encode these proteins on retinal structure and function in vivo in the mouse. SR-BI and ABCA1 expression was assessed by immunofluorescence microscopy in control mouse eyes. ABCA1 was expressed at low levels in the RPE and in most layers of the retina, with high levels of expression in the photoreceptor inner segments (fig 3A). SR-BI was expressed at high levels at the basal

surface of the RPE cell layer with low levels of expression seen in the outer plexiform layer (fig 3B). Each of the immunocytochemical controls, preabsorption with the immunising peptide or omitting the primary antibody, was negative.

The effect of SR-BI and ABCA1 mutation on BrM structure was examined by high-resolution light microscopy. BrM thickness in ABCA1 heterozygous mice appeared to be the same as controls, while SR-BI heterozygotes appeared to show a somewhat thickened BrM. The SR-BI heterozygous mice were then examined by electron microscopy. The BrM of both control mice (figs 4A–C) and SR-BI heterozygous mice (figs 4D and E) showed a heterogeneity of morphological appearances, some with different thickness of RPE and choriocapillaris basement membranes, some with greater or lesser electron lucent vacuoles (fig 4B), and some with greater or lesser thickening of the inner and outer collagenous zones (fig 4A–E). None of these features was consistently different between the SR-BI heterozygotes and control mice. However, mice heterozygous for SR-BI (fig 4D and E) exhibited a modest thickening of BrM compared with controls (figs 4A–C). The mean BrM thickness in SR-BI heterozygous mice was 0.64 (SD 0.01) μm , significantly greater than that in wild-type mice (0.53 (SD 0.01) μm) (unpaired t test $p < 0.0001$).

Retinal function was assessed, both using standard scotopic and photopic flash protocols, and as recovery of scotopic ERG a-wave amplitudes following a 5 min bleach, as described. We detected no scotopic or photopic ERG abnormalities in either SR-BI^{+/-} or ABCA1^{+/-} mice (fig 5A); recovery of dark adaptation after exposure to photobleach was not significantly different between mutant and control mice (fig 5B) (two-way ANOVA $p = 0.16$ for ABCA1^{+/-} and $p = 0.24$ for SR-BI^{+/-} genotype).

DISCUSSION

In AMD, excess lipid accumulation in RPE and BrM is associated with reduced retinal scotopic sensitivity and delayed dark adaptation.¹⁵ This lipid deposition also predisposes patients to severe visual loss from choroidal neovascularisation.¹⁶ We and others have demonstrated similarities between macrophages and RPE with respect to lipid efflux, or RCT.^{9, 15–18} There are also studies indicating important roles for ABCA1 and SR-BI in regulating lipid transport¹⁹ and amyloid protein metabolism²⁰ in the brain. In the current study, we examine expression of these proteins in the retina and RPE.

In previous studies we demonstrated that HDL and purified ApoA-I can stimulate lipid efflux from the basal surface of RPE cells in culture¹¹ and that cultured human RPE cells express SR-BI.⁹ We now demonstrate that ABCA1, an ATP binding cassette transporter that is involved in RCT in other tissues, is expressed by cultured human RPE cells. Importantly, we demonstrate that glyburide, an inhibitor of ABCA1 and SR-BI activity,²¹ inhibited efflux of POS-derived lipids from the basal surface of cultured RPE cells. Thus, RCT in RPE cells in culture is dependent on SR-BI and/or ABCA1 activity. Finally, the present study demonstrates that mouse RPE cells express SR-BI in vivo and that SR-BI^{+/-} mice show increased BrM thickness, although we did not observe a significant effect on retinal function or dark adaptation as measured using ERG. Significantly, we demonstrate that SR-BI is expressed at the basal surface of the mouse RPE in vivo, where it could participate in RCT.

The results suggest that mouse and human eyes may traffic lipids differently. We have reported that human RPE cells express SR-BI.⁹ In the mouse SR-BI appears to be expressed exclusively in the RPE (fig 3). Tserentsoodol *et al* reported expression of SR-BI and SR-BII in both RPE and ganglion cell layers, and ABCA1 in the RPE cell layer in the monkey.²² The observation that SR-BI^{+/-} mice had abnormal BrM thickness, whereas ABCA1^{+/-} mice

had apparently normal BrM, suggests that, in the mouse, SR-BI function may be more important in maintaining normal BrM structure than ABCA1.

One study of ApoE-deficient mice with excess accumulation of lipid in BrM23 showed abnormalities of retinal scotopic sensitivity and recovery from exposure to photobleach.²⁴ However, studies in which ERG abnormalities were present also reported abnormalities of outer and inner retinal structure. We did not find a similar defect in either scotopic retinal function or dark adaptation, perhaps because microscopic studies of mice with heterozygous mutations in ABCA1 and SR-BI did not show any abnormalities of photoreceptor or inner retinal structure. This distinction suggests that BrM thickening, in the absence of photoreceptor abnormalities, is not sufficient to affect either photoreceptor function or dark adaptation.

Because lipid accumulation in the RPE and Bruch's membrane is the hallmark of early AMD, understanding the mechanisms of RCT in these tissues may be critical to developing future therapies for this major cause of visual loss.

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Figure 1. Retinal pigment epithelial cells in culture express ABCA1 mRNA and protein. (A) Panel 1: reverse transcription polymerase chain reaction (RT-PCR) of retinal pigment epithelial (RPE) cell mRNA. DNA molecular weight marker positions are indicated to the left. Panel 2: western blot detection of ABCA1 in RPE cell extracts. Protein molecular weight marker positions are indicated to the right. ABCA1 appears as two bands; a monomer at ~250 kDa and a putative dimer at ~500 kDa. (B) Immunofluorescent detection of ABCA1 protein in RPE cells. Top: staining with anti-ABCA1 antibody. Bottom: control staining with primary antibody omitted.

Figure 2.

Glyburide inhibition of high-density lipoprotein (HDL)-stimulated basal lipid efflux. Basal lipid efflux assays were performed with no addition (CONTROL), with 250 μ mol/l glyburide (GLYB), with 100 μ g/ml HDL (HDL), or with 100 μ g/ml HDL+250 mmol/l glyburide (HDL+GLYB). Total basal ¹⁴C (cpm) is indicated on the y-axis. Results are means and SEM (n=6).

Figure 3.

ABCA1 and SR-BI expression in the wild-type mouse retina. (A) Staining with anti-ABCA1 antibody. Differential interference contrast (DIC) images, with staining with anti-ABCA1 antibody and staining with primary antibody omitted (Ctrl), are shown. (B) Staining with anti-SR-BI antibody. Differential interference contrast (DIC) images, with staining with anti-SR-BI antibody, staining with primary antibody omitted (Ctrl), and staining with anti-SRB-I antibody with inclusion of immunising peptide (Pep.), are shown. GCL, ganglion cell layer. INL, inner nuclear layer; IPL, inner plexiform layer; IS, photoreceptor inner segments; ONL, outer nuclear layer; OS, photoreceptor outer segments; OPL, outer plexiform layer; RPE, retinal pigment epithelium.

Figure 4.

Electron microscopic appearance of Bruch's membranes in control mice (A–C) and SR-BI^{+/-} mice (D–E). The Bruch's membrane of both genotypes displayed varying degrees of thickness of the basement membrane of the retinal pigment epithelium (RPE) foot processes and of the choriocapillaris (CC), as well as differences in the thickness of the collagenous zones. The arrowheads in (A) and (D) show the intervals at which the thickness of Bruch's membrane was measured in the quantitative analysis. Despite some variability in overall thickness of Bruch's membrane in the micrographs, it can be seen in these representative micrographs that it is mostly thinner in the control mice (A–C) than in the SR-BI heterozygotes (D–E). All micrographs are the same magnification. Scale bar, 0.5 μ m.

Figure 5. ABCA1^{+/-} and SR-BI^{+/-} deficit had no effect on the ERG or dark adaptation in adult mice. (A) Representative scotopic ERG responses from control (left panel), ABCA1^{+/-} (right panel, top) and SR-BI^{+/-} mutant mice (right panel, bottom). Arrow indicates time of stimulus. (B) Recovery of photoreceptor function was not significantly different from control in mice with heterozygous ABCA1 or SR-BI mutations (two-way ANOVA $p=0.16$ for ABCA1^{+/-} and $p=0.24$ for SR-BI^{+/-} genotype). Scotopic a-wave amplitudes after exposure to a photobleach of 29 cd/m² for 5 min were recorded at 5 min intervals in response to a flash of +0.4 log cd/m² over a recovery period of 60 min; a-wave amplitudes were measured 10 ms after the stimulus, prior to intrusion of post-receptoral responses, and were normalised by dividing by the pre-bleach a-wave amplitude.