Reduction of lipid accumulation rescues Bietti’s crystalline dystrophy phenotypes

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Bietti’s crystalline dystrophy (BCD) is an intractable and progressive chorioretinal degenerative disease caused by mutations in the CYP4V2 gene, resulting in blindness in most patients. Although we and others have shown that retinal pigment epithelium (RPE) cells are primarily impaired in patients with BCD, the underlying mechanisms of RPE cell damage are still unclear because we lack access to appropriate disease models and to lesion-affected cells from patients with BCD. Here, we generated human RPE cells from induced pluripotent stem cells (iPSCs) derived from patients with BCD carrying a CYP4V2 mutation and successfully established an in vitro model of BCD, i.e., BCD patient-specific iPSC-RPE cells. In this model, RPE cells showed degenerative changes of vacuolated cytoplasm similar to those in postmortem specimens from patients with BCD. BCD iPSC-RPE cells exhibited lysosomal dysfunction and impairment of autophagy flux, followed by cell death. Lipidomic analyses revealed the accumulation of glucosylceramide and free cholesterol in BCD-affected cells. Notably, we found that reducing free cholesterol by cyclodextrins or δ-tocopherol in RPE cells rescued BCD phenotypes, whereas glucosylceramide reduction did not affect the BCD phenotype. Our data provide evidence that reducing intracellular free cholesterol may have therapeutic efficacy in patients with BCD.

Significance

Bietti’s crystalline dystrophy (BCD) is an autosomal recessive, progressive chorioretinal degenerative disease. Retinal pigment epithelium (RPE) cells are impaired in patients with BCD, but the underlying mechanisms of RPE cell damage have not yet been determined because cells from lesions cannot be readily acquired from patients with BCD. In the present study, we successfully generated a human in vitro model of BCD, BCD patient-specific iPSC-RPE cells, and demonstrated that the accumulation of free cholesterol caused RPE cell damage and subsequent cell death via the induction of lysosomal dysfunction and impairment of autophagy flux in BCD-affected cells. We believe these findings provide evidence of the possible therapeutic efficacy of reducing intracellular free cholesterol in BCD.

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phenotypes and lipid profiles of BCD patient-specific iPSC-RPE cells to investigate the mechanisms underlying the onset and progression of BCD. In addition, we sought to identify compounds that could rescue BCD-associated phenotypes.

Results

Generation of BCD Patient-Specific iPSCs and iPSC-Derived RPE Cells. We established iPS lines from three BCD patients (BCD-1, BCD-2, and BCD-3) (Fig. S1) that carried the homozygous mutation indel c.802-8_810del17insGC (CYP4V2 mut1) (Fig. S1) (7) in the CYP4V2 gene and normal control (NOR) iPS lines derived from three control individuals with normal fundus and without CYP4V2 gene mutations. There were no remarkable differences between BCD and NOR iPS lines during the establishment of iPSC preparations.

Thereafter, we induced BCD and NOR iPSCs to differentiate toward RPE cells (14). Differentiated iPSC-RPE cells with a polygonal, cobblestone-like morphology were cultured for over 90 d until high pigmentation appeared that indicated full functional maturity (Fig. 1A). The mRNA and protein levels of key RPE biomarkers, CRALBP and RPE65, were significantly elevated in differentiated RPE cells from both NOR and BCD iPSCs compared with undifferentiated iPSC cells (Fig. S2). Notably, light microscopic examination revealed disturbances of BCD iPSC-RPE cell arrangement (Fig. 1A), as was confirmed with ZO-1 staining (Fig. 1B). Whereas the CYP4V2 protein was detected in NOR iPSC-RPE cells, it was not detected in BCD iPSC-RPE cells (Fig. 1C) despite similar CYP4V2 mRNA expression levels in NOR and BCD iPSC-RPE cells (Fig. S3). Furthermore, despite the redundancy in the activity of CYP4 family members (15), the mRNA expression levels of other genes from CYP4 family did not change in BCD iPSC-RPE cells compared with the levels seen in NOR iPSC-RPE cells (Fig. S3), indicating the absence of obvious compensatory changes in the expression of other CYP4 family members.

Morphological and Functional Analyses of BCD iPSC-RPE Cells. Remarkably, with time in culture, degenerative changes, such as vacuole formation, larger cell size, and pronounced pigmentation changes, were observed by phase-contrast microscopy in all lines of BCD iPSC-RPE cells (Fig. 1D). Compared with NOR iPSC-RPE cells, degenerative cells exhibiting all the following features: vacuole formation, larger cell size, and pronounced pigmentation changes were seen significantly more frequently among BCD iPSC-RPE cells (Fig. 1E). Transmission electron microscopy (TEM) observations confirmed that NOR iPSC-RPE cells grew as a monolayer of highly polarized cells with abundant apical microvilli and melanosomes, whereas BCD iPSC-RPE cells exhibited accumulations of melanosomes and autophagosomes and intracellular pigmented granules and vacuolated cytoplasm (Fig. 1F) that recapitulated RPE changes reported in BCD patients (16). To better characterize the phenotype of iPSC-RPE cells, we evaluated the growth rate and cell death rate of iPSC-RPE progenitor cells (Materials and Methods). Compared with NOR iPSC-RPE progenitor cells, BCD iPSC-RPE progenitor cells showed a significantly lower rate of cell growth (Fig. 1G). To evaluate the state of cell proliferation, the numbers of Ki67+/DAPI+ cells were counted, and the percentage of Ki67+/DAPI− cells was evaluated. BCD iPSC-RPE progenitor cells showed a lower percentage of Ki67+ cells in the DAPI+ population (Fig. 1H and I). Furthermore, we found a greater rate of cell death in BCD iPSC-RPE progenitor cells than in NOR iPSC-RPE progenitor cells (Fig. 1J).

To determine whether the cellular phenotypes observed in BCD iPSC-RPE cells were caused by the loss of function of the CYP4V2 gene, we transferred wild-type CYP4V2 (hereafter, CYP4V2 WT), BCD mutant CYP4V2 (hereafter, CYP4V2 mut1), or a mock sequence (control) into BCD iPSC-RPE cells by a recombinant adenovirus vector (Fig. S4). As expected, the CYP4V2 WT protein level increased only in BCD iPSC-RPE cells infected with CYP4V2 WT, and the CYP4V2 mut1 protein level increased only in those infected with CYP4V2 mut1 (Fig. S4B). CYP4V2 WT or mutant mRNA levels were elevated in BCD iPSC-RPE cells infected with CYP4V2 WT and CYP4V2 mut1 but not in those infected with the mock sequence (Fig. S4C). Compared with BCD iPSC-RPE cells infected with the mock sequence (control) or CYP4V2 mut1, BCD iPSC-RPE cells infected with CYP4V2 WT showed a significantly lower rate of degenerative changes, which were defined as described above (Fig. S4 A and D), and a higher rate of cell growth (Fig. S4E), indicating that the phenotypes observed in BCD iPSC-RPE cells were caused by the loss of function of the CYP4V2 gene. To investigate whether other types of CYP4V2 gene mutations may cause BCD phenotypes, we generated HEK293 cells with mutated CYP4V2 using the CRISPR/Cas9 system. The introduced mutation (hereafter, CYP4V2 mut2) caused a frame shift within exon 5 so that the mutated gene would encode only a very short protein (218 aa) (Fig. S4). As expected, these CYP4V2-mutated HEK293 cells showed vacuole formation and subsequently had a higher death rate, i.e., degenerative changes similar to those observed in BCD iPSC-RPE cells (Fig. S4 H–K). Furthermore, transfection with CYP4V2 WT adenovirus vector rescued the cellular phenotypes observed in CYP4V2-mutated HEK293 cells (Fig. S4 L and M).

Mechanisms of Cellular Damage in BCD iPSC-RPE Cells. Next, we investigated the mechanisms of degenerative changes found in BCD iPSC-RPE cells. From the findings of TEM examination, autophagy was suspected to be impaired in BCD. Therefore, we first evaluated the expression of the autophagy marker, microtubule-associated protein 1A/1B light chain 3 (LC3), in BCD iPSC-RPE cells infected with CYP4V2 WT or mutant mRNA levels were elevated in BCD iPSC-RPE cells infected with CYP4V2 WT and CYP4V2 mut1 but not in those infected with the mock sequence (Fig. S4C). Compared with BCD iPSC-RPE cells infected with the mock sequence (control) or CYP4V2 mut1, BCD iPSC-RPE cells infected with CYP4V2 WT showed a significantly lower rate of degenerative changes, which were defined as described above (Fig. S4 A and D), and a higher rate of cell growth (Fig. S4E), indicating that the phenotypes observed in BCD iPSC-RPE cells were caused by the loss of function of the CYP4V2 gene. To investigate whether other types of CYP4V2 gene mutations may cause BCD phenotypes, we generated HEK293 cells with mutated CYP4V2 using the CRISPR/Cas9 system. The introduced mutation (hereafter, CYP4V2 mut2) caused a frame shift within exon 5 so that the mutated gene would encode only a very short protein (218 aa) (Fig. S4). As expected, these CYP4V2-mutated HEK293 cells showed vacuole formation and subsequently had a higher death rate, i.e., degenerative changes similar to those observed in BCD iPSC-RPE cells (Fig. S4 H–K). Furthermore, transfection with CYP4V2 WT adenovirus vector rescued the cellular phenotypes observed in CYP4V2-mutated HEK293 cells (Fig. S4 L and M).

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proteins of the LC3 and p62 pathways (Fig. 2 A and B). Expression of LC3-II and p62 with or without bafilomycin-A1 (Baf A1, 20 nM) in NOR and BCD iPSC-RPE cells. NOR Tx− vs. NOR with Baf A1: * = 0.0459 (LC3-II) and * = 0.0496 (p62), paired t test, n = 3 in each group; NOR Tx− vs. BCD Tx+: ***P < 0.001 (LC3-II) and **P = 0.0013 (p62), Student’s t test; n = 3 in each group. (C and D) Expression of LAMP1 and LAMP2. *P < 0.05; Student’s t test; n = 3 derived from each of three lines. (E) Immunocytochemical staining for LAMP2 (green) and ZO1 (red). (Scale bars: 10 μm.) (F and G) FACs analysis of lysosome function using LysoTracker Green. ***P < 0.001, BCD vs. NOR; Student’s t test; n = 3 derived from each of three lines. (H) Lysosomal pH measurement using LysoSensor. The F340/380 ratio was determined. *P = 0.0495, BCD vs. NOR; Student’s t test; n = 3 in each group. (I) The cathepsin D activity levels measured using a fluorometric cathepsin D activity assay. *P = 0.032, BCD vs. NOR; Student’s t test; n = 3 derived from each of three lines. Error bars indicate SD.

Fig. 2. Autophagy and lysosome function in NOR and BCD iPSC-RPE cells. (A and B) Expression of LC3-II and p62 with or without bafilomycin-A1 (Baf A1, 20 nM) in NOR and BCD iPSC-RPE cells. NOR Tx− vs. NOR with Baf A1: * = 0.0459 (LC3-II) and * = 0.0496 (p62), paired t test, n = 3 in each group; NOR Tx− vs. BCD Tx+: ***P < 0.001 (LC3-II) and **P = 0.0013 (p62), Student’s t test; n = 3 in each group. (C and D) Expression of LAMP1 and LAMP2. *P < 0.05; Student’s t test; n = 3 derived from each of three lines. (E) Immunocytochemical staining for LAMP2 (green) and ZO1 (red). (Scale bars: 10 μm.) (F and G) FACs analysis of lysosome function using LysoTracker Green. ***P < 0.001, BCD vs. NOR; Student’s t test; n = 3 derived from each of three lines. (H) Lysosomal pH measurement using LysoSensor. The F340/380 ratio was determined. *P = 0.0495, BCD vs. NOR; Student’s t test; n = 3 in each group. (I) The cathepsin D activity levels measured using a fluorometric cathepsin D activity assay. *P = 0.032, BCD vs. NOR; Student’s t test; n = 3 derived from each of three lines. Error bars indicate SD.

Optimal lysosome function requires the ability to maintain acidic pH, and LysoTracker Green is a lysosomotropic dye that permits monitoring of pH-sensitive indices of the lysosomal function (21). We found that BCD iPSC-RPE cells showed a loss of LysoTracker signal compared with similar observations in NOR iPSC-RPE cells (Fig. 2 F and G). Lysosomal pH measurements using LysoSensor showed that the F340/380 ratio of fluorescence excited at 340 nm and 380 nm was higher (indicating a more alkaline state) in BCD iPSC-RPE cells than in NOR iPSC-RPE cells, indicating lysosomal alkalization in BCD pathology (Fig. 2H). Further, the activity of cathepsin D, the primary aspartyl protease of the lysosome that has a particularly acidic pH optimum and pH-dependent maturation (22, 23), was impaired in BCD iPSC-RPE cells (Fig. 2F). Taken together, our findings indicate that the autophagic degradative system may be disturbed as a result of lysosomal dysfunction in BCD.

Lipidomic Analyses in BCD iPSC-RPE Cells. Because the CYP4V2 protein was predicted to be involved in lipid metabolism as a member of the cytochrome P450 superfamily, we performed comprehensive liqiue choromatography (LC)-MS/MS–based lipidomic analyses. First, we evaluated the enzyme activity of CYP4V2 as a hydroxylase of polyunsaturated fatty acids (PUFAs) by using HEK293 cells overexpressing CYP4V2 WT, CYP4V2 mut1, and CYP4V2 mut2. LC-MS/MS–based lipidomics showed that CYP4V2 WT protein possessed α-oxygenase and ω-1-hydroxylation activities (Fig. S6A), consistent with the previous report (9). However, the levels of those PUFAs metabolites were under the detection limit in NOR and BCD iPSC-RPE cell culture media (Fig. S6B). Next, we performed untargeted lipidomics of NOR and BCD iPSC-RPE cells and found the accumulation of various glucosylceramides (GlcCer) and the reduction of cholesteryl esters in BCD iPSC-RPE cells (Fig. 3 A and B). We also determined the accumulation of free cholesterol per cell number in BCD iPSC-RPE cells (Fig. 3C). Filipin staining confirmed free cholesterol enrichment in BCD iPSC-RPE cells compared with NOR iPSC-RPE cells (Fig. 3D). These lipid profiles were similar to those observed in several types of lysosomal storage diseases, e.g., Niemann-Pick type C (NPC) or Gaucher’s disease (24, 25). We also examined the mRNA expression levels of the causative genes of lysosomal storage diseases, genes involved in the metabolism/synthesis of glycolipids, and genes regulating intracellular cholesterol transport. NPC1 was slightly up-regulated in BCD iPSC-RPE cells, but the expression levels of other genes did not differ between NOR and BCD iPSC-RPE cells (Fig. S5).

Dysfunctional reverse cholesterol transport due to abnormal CYP27A1, CYP46a1, and CYP11A1 expression and LXR metabolism

is a well-known cause for retinal and RPE cell pathology (26–29). However, oxysterol levels did not differ significantly between NOR and BCD iPSC-RPE cells (Fig. S6C). Furthermore, mRNA levels of CYP27A1, CYP46A1, CYP11A1, and LXRβ did not differ between NOR and BCD iPSC-RPE cells (Fig. S5). Therapeutic Effect of Lipid Accumulation Reduction on Phenotypes in BCD iPSC-RPE Cells. Because the accumulations of GlcCer and free cholesterol were observed in BCD iPSC-RPE cells, we investigated the effects of the reduction of lipid accumulation on BCD phenotypes. We tested several compounds, including cyclohexatrins (CDs) (30–34), statinlovastatin, and δ-T on intracellular free cholesterol (A) or cholesteryl ester levels (B) in BCD iPSC-RPE cells derived from three lines. ***P < 0.001, **P < 0.01, *P < 0.05 vs. no treatment [Tx; one-way ANOVA followed by the Dunnett’s test; n = 3. (C and D) LC-MS/MS-based lipidomic analyses of therapeutic effects of NBDNJ and CDs (HPBCD and HPGCD) on cholesteryl ester (C) or GlcCer (D). *P < 0.05, **P < 0.01, ***P < 0.001 vs. no treatment [Tx; one-way ANOVA followed by the Dunnett’s test; n = 3].

Fig. 4. Effect of CDs on lipids in BCD iPSC-RPE cells. (A and B) Therapeutic effects of NBDNJ, cyclohexatrins (CDs), lovastatin, and δ-T on intracellular free cholesterol (A) or cholesteryl ester levels (B) in BCD iPSC-RPE cells derived from three lines. ***P < 0.001, **P < 0.01, *P < 0.05 vs. no treatment [Tx]; one-way ANOVA followed by the Dunnett’s test; n = 3. (C and D) LC-MS/MS-based lipidomic analyses of therapeutic effects of NBDNJ and CDs (HPBCD and HPGCD) on cholesteryl ester (C) or GlcCer (D). *P < 0.05, **P < 0.01, ***P < 0.001 vs. no treatment [Tx]; one-way ANOVA followed by the Dunnett’s test; n = 3.

Next, we investigated the therapeutic effects of these compounds on cellular phenotypes of BCD iPSC-RPE cells. HPBCD and HPGCD, which reduced free cholesterol per cell number, suppressed the development of degenerative changes in BCD iPSC-RPE cells. This result was also supported by TEM examination, which showed a significant number of enlarged lysosomes with osmophilic structures in a clear matrix in nontreated BCD iPSC-RPE cells, but the amount of these hallmark structures in the cytosol was substantially lower in BCD iPSC-RPE cells treated with HPBCD (Fig. 5C). HPBCD and HPGCD also improved the cell growth rate and attenuated the cell death rate in BCD iPSC-RPE progenitor cells (Fig. 5D and E). In contrast, NBDNJ, which reduced GlcCer but not free cholesterol levels per cell number, did not improve these cellular phenotypes. We also found that HPBCD and HPGCD tended to suppress the expression levels of LC3-II and p62 proteins in BCD iPSC-RPE cells, whereas NBDNJ was without effect (Fig. 5F and Fig. S7). In addition, HPBCD and HPGCD improved the Lysotracker signal in BCD iPSC-RPE cells, whereas NBDNJ did not (Fig. 5G and Fig. S7). In fact, HPBCD treatment decreased lyosomal pH in BCD iPSC-RPE cells as detected using Lysosensor; the improvement rates in the F430/380 ratio in BCD iPSC-RPE cells, defined as [BCD each line (Tx–) − BCD each line (HPBCD)]/[BCD each line (Tx–) − NOR3 lines average], where Tx represents no treatment, were 67.7%, 80.0%, and 159.4% in the BCD-1, BCD-2, and BCD-3 cell lines, respectively. This indicated that the lysosomal alkalinization observed in the BCD iPSC-RPE cells was improved by HPBCD treatment. Thus, in agreement with the results of lipodics, HPBCD and HPGCD reversed autophagic flux impairment and lysosomal dysfunction, but NBDNJ did not. These results imply that the cellular phenotypes observed in BCD iPSC-RPE cells were caused not by the accumulation of...
GlcCer but by the increase of free cholesterol level. On the contrary, HPBCD did not affect the percentages of degenerative cell and cell death or lysosome function in NOR iPSC-RPE cells (Fig. S8).

Discussion

In this study, we generated human iPSC-RPE cells derived from BCD patients carrying a CYP4V2 mutation and successfully established a human in vitro model of BCD. In this model, RPE cells showed degenerative changes of vacuolated cytoplasm, i.e., a typical cellular phenotype observed in BCD. Using BCD iPSC-RPE cells, we revealed that in BCD the accumulation of free cholesterol was associated with lysosomal dysfunction due to lysosomal alkalization and impairment of autophagy flux and thereby caused RPE cell damage followed by cell death. Based on this pathophysiology, we discovered that the reduction of free cholesterol in RPE cells rescued BCD phenotypes, which suggested that compounds that decrease the accumulation of free cholesterol could be therapeutic agents for BCD.

The difficulty in accessing human RPE and a lack of appropriate disease models have hampered efforts to investigate the pathology of BCD and to develop new drug candidates for this disease. The reported murine model for BCD (10) is not an entirely appropriate human BCD model for the following reasons. First, mice with the knockout of the Cyp4v3 gene (the murine ortholog of CYP4V3, not CYP4V2 itself) mainly exhibit impairment of photoreceptors but not of RPE, whereas clinical findings suggest that RPE is the primary lesion site in BCD (4, 6). Second, the chorioretinal impairment in these mutant mice is milder than that in human BCD. For understanding the underlying mechanism, HEK293 cells harboring the CYP4V2 knockout were expected to be useful because of the degenerative changes observed in the mutant. However, a sufficient number of these mutant cells could not be collected for analysis of lipid profiles, as the CYP4V2 knockout lowers the growth rate. In our experiments, BCD iPSC-RPE cells served as a disease model, as they recapitulated the cellular phenotypes similar to those of NPC (46, 47), the disturbances in the metabolism of steroids. Given that several steroids have in- water solubility and a hydrophobic interior that accommodates free cholesterol accumulation was a limitation of our study. CYP

Among the compounds we tested, several CDs (HPBCD, HPGCD, and MBCD) and δ-T had positive therapeutic effects on the accumulation of free cholesterol. CDs, which are cyclic oligosaccharides composed of six to eight glucopyranosides, have a distinct barrel configuration with a hydrophilic exterior promoting water solubility and a hydrophobic interior that accommodates small lipophilic molecules. Some CDs have been reported to reduce the accumulation of free cholesterol and to increase life span in NPC models, possibly by working as a shuttle facilitating the egress of the trapped free cholesterol to intracellular sites for normal sterol processing (30-32, 34). In contrast, δ-T, a minor vitamin E species, appears to exert its effect in NPC models through the stimulation of lysosomal exocytosis (36). Importantly, although we observed that CDs and δ-T had opposite effects on cholesterol ester levels, these compounds improved cellular phenotypes of BCD, indicating that therapies lowering intracellular free cholesterol could provide therapeutic benefit for BCD patients. With relevance to this point, HPBCD and HPGCD are currently in clinical trials as NPC treatments (33, 34). Thus, these are potential drug candidates for future treatment of BCD.

The lack of a proven mechanism linking CYP4V2 function and free cholesterol accumulation was a limitation of our study. CYP proteins have multiple functions, one of which is involvement in the metabolism of steroids. Given that several steroids have inhibitory effects on lysosomal cholesterol transport, resulting in phenotypes similar to those of NPC (46, 47), the disturbances in CYP4V2 protein functions may affect lysosomal cholesterol transport via sterol metabolism impairments. The other limitation was the potential influence of cell size on intracellular cholesterol content. The free cholesterol concentration per cell number was higher in BCD iPSC-RPE cells than in NOR iPSC-RPE cells. In contrast, normalization to protein content (owing to the larger RPE cell size in BCD pathology) could not clearly demonstrate whether free cholesterol content was significantly higher in BCD iPSC-RPE cells (Fig. S9B). Nevertheless, we
believe that higher content of free cholesterol per cell number is associated with BCD pathophysiology for the following reasons: (i) the cholesterol ester level apparently decreased in BCD iPS-RPE cells; (ii) free cholesterol enrichment was demonstrated by flow cytometry of unknown localization; and (iii) treatments with HPCD/HPGC decreased free cholesterol content per cell in BCD iPS-RPE cells, which corresponded to the improvements in BCD phenotype.

In summary, we successfully generated a human in vitro model of BCD, BCD iPS-RPE cells, and unveiled the following mechanisms of cellular damage in BCD RPE cells: (i) the accumulation of free cholesterol that is associated with lysosomal impairments and (ii) lysosomal dysfunction that impaired autophagy flux and led to the higher extent of RPE degenerative changes and cell death. Our data also provided evidence of the possible therapeutic efficacy of intracellular free cholesterol reduction for BCD patients.

Materials and Methods

This study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Kyoto University Graduate School of Medicine (2016:2018). After informed consent was obtained from three BCD patients with the homozygous mutation c.802_B.810del17insGC in the CYP4V2 gene and from three control individuals with normal fundus and without CYP4V2 gene mutations, fibroblast cell lines were generated. The method used for human iPS generation was described previously (48). We analyzed three fibroblast cell lines (BCD-1 (CYP4V2-9909-G), BCD-2 (CYP4V2-9909-G), and BCD-3 (CYP4V2-9905-B)) and three cell lines of NOR controls (NOR-1 (CYP4V2-9902-A), NOR-2 (CYP4V2-9156-A), and NOR-3 (CYP4V2-9157-A)).

Additional methods can be found in SI Materials and Methods.

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