En face slab optical coherence tomography imaging successfully monitors progressive degenerative changes in the innermost layer of the diabetic retina

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ABSTRACT

Objective To evaluate the usefulness of en face slab optical coherence tomography (OCT) imaging for monitoring diabetic retinal neurodegeneration with supporting animal experimental data.

Research design and methods We retrospectively examined 72 diabetic eyes over 3 years using Cirrus-HD OCT. Two-dimensional en face slab OCT images of the innermost retina were reconstructed and graded according to the ratio of dark area to total area, and relative red, green, and blue color area ratios were calculated and used as indexes for each en face slab OCT image. Values from en face OCT images were used for statistical analyses. To obtain insight into the pathogenesis of diabetic retinal neurodegeneration, we used the *InsPr-Cre;Pdk1*^flox/flox diabetic mouse model.

Results Both OCT grade and relative red color area ratio significantly increased with the advancing stage of diabetic retinopathy (p=0.018 and 0.006, respectively). After a mean follow-up period of 4.6 years, the trend was unchanged in the analyses of 42 untreated eyes (p<0.001 and 0.001, respectively). Visual acuity showed a weak but significant negative correlation with the red color ratio on en face slab OCT images, but central retinal thickness did not exhibit a clinically meaningful correlation with values obtained from en face slab OCT images. Immunohistochemical analyses of *InsPr-Cre;Pdk1*^flox/flox diabetic mice demonstrated the loss of ganglion axon bundles and thinning of laminin without apparent retinal vascular change at the age of 20 weeks.

Conclusions En face slab OCT imaging would be a novel useful modality for the assessment of diabetic retinal neurodegeneration as it could detect subtle optical changes occurring in the innermost retina in diabetic eyes. Our animal experimental data suggest that dark areas observed on en face slab OCT images might be the impairment of the extracellular matrix as well as neurons.

Significance of this study

What is already known about this subject?

► Diabetic retinopathy (DR) has been classified based on clinical signs secondary to severely impaired retinal vasculatures. Previous studies show that histological and/or functional retinal changes occur even before the onset of clinically detectable microvascular damage.

What are the new findings?

► En face slab optical coherence tomography (OCT) images worsened with the advancing stage of DR. Progressive reflectance alterations of the innermost layer over the years were observed on en face slab OCT images. Animal experimental data suggest that dark areas observed on en face slab OCT images might be the impairment of the extracellular matrix as well as neurons.

How might these results change the focus of research or clinical practice?

► Our findings indicate that en face slab OCT imaging, which represents a subtle change in optical property of the innermost retina, could provide a novel information on diabetic retinal neurodegeneration probably caused by neuronal cell death and decreased extracellular matrix deposition. En face slab OCT imaging might contribute to early detection and long-term monitoring of diabetic retinal damage in clinical practice.

INTRODUCTION

With the increasing prevalence of diabetes mellitus (DM) worldwide, how to reduce the burden of diabetes-related complications is becoming a global concern.[1] Diabetic retinopathy (DR) is the most common diabetes complication and one of the major causes of severe visual impairment, leading to considerable socioeconomic cost. Because pathological changes in diabetic retinas are considered irreversible, prevention, early diagnosis, and

early intervention are the best strategies to preserve life-long vision for diabetic patients. Although accumulating evidence suggest that the eyes of patients with DM can have histological and/or functional retinal changes even before the onset of clinically detectable microvascular damage,[2–4] the gold standard classification of DR is based on funduscopically apparent clinical signs secondary to severely impaired retinal vasculature.[5] The Early Treatment Diabetic Retinopathy Study classification according to vascular abnormality has been used in many randomized controlled trials (RCTs). However, although one intervention (intensive blood sugar control) decreased the risk of DR development and/or progression, there remains a large number of patients with vision loss in almost all RCTs, suggesting that funduscopy-based DR classification is not sufficient to maintain long-term good vision in diabetic eyes.[6–8]

A large body of evidence suggest that diabetes-induced retinal changes occur in both vascular and non-vascular cell types[9 10] and that retinal neurodegeneration is an early event in the DR pathogenesis, prior to obvious manifestations of microvascular abnormalities.[2 3] Many clinical attempts have been made to detect neuronal retinal changes in diabetic eyes, most of which focused on the abnormalities of the inner retina because it is prone to damage under diabetic conditions.[11–15] Functional testing, such as multifocal electroretinography (mfERG), is an ideal method of objective evaluation of diabetic neuronal damage.[16] However, this method is generally time-consuming and therefore difficult to perform in a routine examination. Spectral domain optical coherence tomography (OCT) is commonly used for structural assessment of inner retinal pathology, as it provides high-quality three-dimensional (3D) information on retinal pathology in a rapid and non-invasive manner. In previous clinical studies of diabetic retinal neurodegeneration, most investigators used structural properties, measuring the thickness of the total retina, the retinal nerve fiber layer (RNFL), or the macular ganglion cell layer–inner plexiform layer (mGCIPL),[11 12 15 17] whereas little attention was paid to the optical properties of retinal tissue (eg, strength of the OCT signal), although both structural and optical changes can be expected to occur during the process of neurodegeneration.

Hood *et al* derived en face slab OCT images from obtained 3D OCT data and showed that en face slab OCT imaging can display details of RNFL abnormalities not seen on traditional OCT RNFL thickness analysis.[18] Our group successfully assessed the surgical damage to the innermost retina after internal limiting membrane (ILM) peeling,[19 20] and Miwa *et al* used it for the evaluation of diabetic retinal vascular changes.[21] Thus, evidence is growing that en face slab OCT imaging enables assessment of optical changes in the retina. In this study, we investigated en face slab OCT images of the diabetic retina and compared the images among different stages of DR. We also assessed the changes in the images over the years. Further, we conducted animal experiments to evaluate what elements of the inner retinal layer are impaired during the process of diabetic retinal neurodegeneration. These results may influence OCT analyses of retinal structures and further help us understand the differences between the changes in thickness and optical properties in the OCT assessment of the retina.

RESEARCH DESIGN AND METHODS
We retrospectively reviewed all of the medical records of diabetic patients who underwent Cirrus HD-OCT imaging (Cirrus HD-OCT model 4000, Carl Zeiss Meditec, Dublin, California, USA) at Kobe University Hospital between September 2008 and September 2014. All images were obtained by an experienced technician using the 200×200 macular cube protocol and were automatically saved as cube scan data. To be included in this study, each patient had to meet the following criteria: having a diagnosis of diabetes and information on DR, regularly followed up for more than 3 years, and having accessible Cirrus HD-OCT data over a time interval of more than 3 years. The exclusion criteria were the presence of an apparent diabetic macular edema (DME) (defined as central retinal thickness (CRT)>350 µm), vitrectomized eyes, any intraocular surgery during the follow-up period, and a history of non-diabetic ocular pathologies that might affect the properties of retinal tissue. OCT images with artifacts, segmentation failure, or poor image quality (signal strength <6) were also excluded. The collected demographic and clinical data were age, gender, eye laterality, axial length, lens status, DR stage according to the International Clinical Diabetic Retinopathy Disease Severity Scale,[5] best-corrected visual acuity (BCVA) and intraocular pressure at the time of OCT testing, Cirrus HD-OCT 200×200 cube scan data (CRT, mGCIPL thickness, and macular volume) obtained as previously reported,[17] follow-up period, and treatment for DR during the follow-up period.

En face slab OCT imaging and analysis
En face slab OCT images of the innermost retina were reconstructed from the cube scan data. In brief, by selecting the 'ILM' tab from the drop-down menu in the advanced visualization mode, the onboard Cirrus OCT software uses a built-in algorithm and automatically creates an en face image of a slab beneath the vitreous/ILM boundary with a default thickness of 20 µm. An en face slab OCT image is composed of 200×200 pixels and is displayed as a color-coded reflectance map covering a 6×6 mm area centered on the fovea. As the color of each pixel represents an average OCT reflectivity value for each A-scan location, en face slab OCT imaging allows a straightforward assessment of the tissue properties of the innermost retina. We graded two-dimensional (2D) en face slab OCT images from 0 to 3 according to the ratio of dark area to total area (grade 0<1/4, grade 1≥1/4 and <1/2, grade 2≥1/2 and <3/4, grade 3≥3/4) (figure 1A–D).
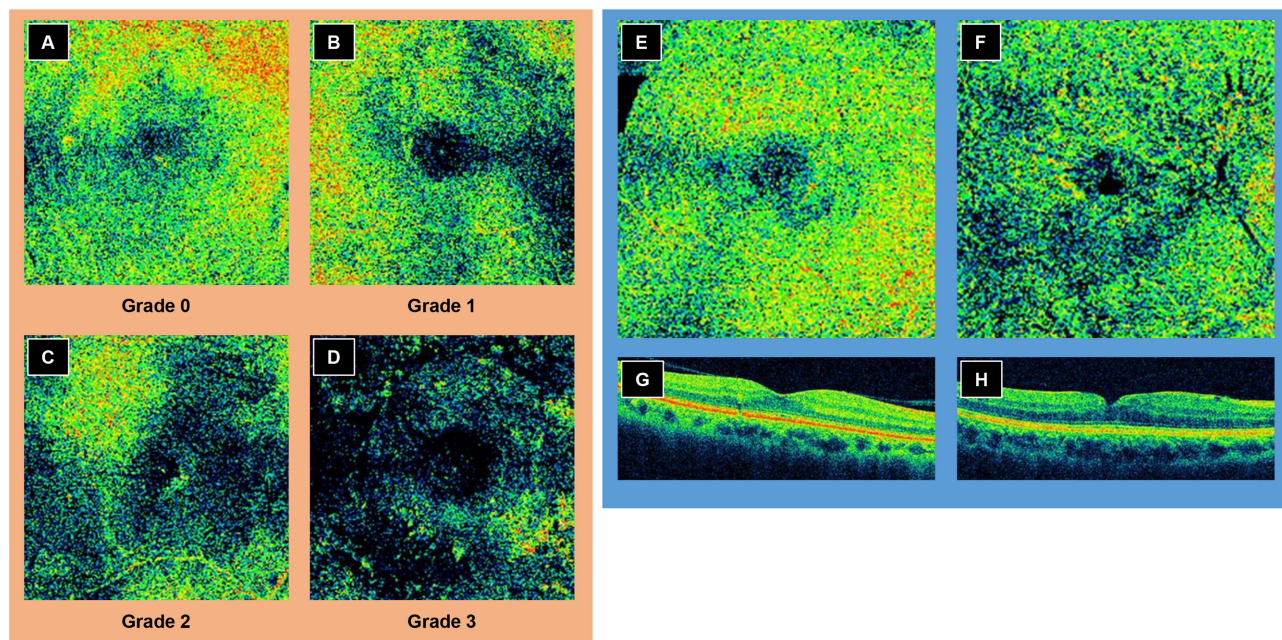
Figure 1 Grading of en face slab OCT images. (A–D) En face slab OCT images were graded from 0 to 3 according to the ratio of dark area to total area (grade 0<1/4, grade 1≥1/4 and <1/2, grade 2≥1/2 and <3/4, grade 3≥3/4). (E–H) Representative case of a patient with diabetes mellitus who underwent long-term follow-up by en face slab OCT imaging. (E) There was a modest dark area on en face slab OCT image at baseline. (F) The dark area was diffusely enlarged after a 4-year follow-up. (G,H) The change in two-dimensional en face slab OCT image over time was not detectable by section OCT image. OCT, optical coherence tomography.

For a detailed analysis of en face slab OCT images, we used ImageJ software (https://imagej.nih.gov/ij/). In brief, each image was separated into three bands of color components (red, green, and blue), and each color image was converted to a grayscale image. Then, the signal area in this grayscale image was measured. Consequently, a measurement value for each color area was obtained. We used the color area ratio as an index of the structural status of the inner retina because normal inner retinas are known to have a highly scattering optical property, and damaged inner retinas would lose the property.[18 22 23] In other words, it is expected that normal and damaged retinas have red and blue color areas, respectively. In this study, the mean color area ratios at different stages of DR were compared using baseline en face slab OCT images, and the mean changes in color area ratio over time (>3 years) were analyzed.

Animal experiments

This animal study was approved by the Kobe University Animal Care and Use Committee (permission number P160207) and was carried out according to the Kobe University Animal Experimentation Regulations and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Hyperglycemia was produced in mice as follows. *InsPr-Cre* transgenic and $Pdk1^{flox/+}$ knockout mouse lines were generated previously.[24 25] We backcrossed these mice into the C57BL/6J background (purchased from CLEA Japan, Tokyo, Japan) for more than 10 generations. Then, we generated pancreatic β cell-specific $Pdk1$ knockout mice ($InsPr-Cre^{Tg/+}$; $Pdk1^{flox/flox}$ [β$Pdk1^{-/-}$]) by mating $Pdk1^{flox/+}$ mice with $InsPr-Cre^{Tg/+}$ mice.[26] All of the mice were maintained under standard laboratory conditions (12/12 hour light/dark cycle and 20°C–24°C; food and water were provided without restriction). For all procedures, anesthesia was achieved by intraperitoneal injection of a mixture of 0.75 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol. Male mice were used in the experiments. Blood glucose levels in the non-fasting state were measured every 4 weeks, and mice with blood glucose levels of >350 mg/dL at 8 weeks of age were considered diabetic. Diabetic β$Pdk1^{-/-}$ mice and control $Pdk1^{flox/flox}$ mice were sacrificed for immunohistochemical analyses at 20 weeks of age.

Retinal whole-mount and section immunohistochemistry (IHC) was performed as previously reported.[27 28] The primary and secondary antibodies used were Alexa Fluor 488 conjugated anti-Tubulin β3 antibody (Invitrogen), anti-Laminin antibody (Sigma-Aldrich, St. Louis, Missouri, USA), anti-CD31 antibody (BD Pharmingen, San Diego, California, USA), anti-alpha-Smooth Muscle Actin (αSMA) antibody (Sigma-Aldrich), Cy3 conjugated anti-Glial Fibrillar Acidic Protein (GFAP) antibody (Sigma-Aldrich), anti-Fibronectin antibody (DAKO, Copenhagen, Denmark), anti-Type 4 collagen (Cosmo Bio, Tokyo, Japan), Cy3 conjugated anti-rabbit IgG antibody (Sigma-Aldrich), Alexa Fluor 488 conjugated anti-rat IgG antibody (Invitrogen), and TO-PRO-3 (Invitrogen).

We used ImageJ software for quantitative analyses. Ganglion cell axons on retinal flat mounts were quantified

from four randomly selected ×10 fluorescence micrographs from central regions at approximately 100 µm from the optic disc. The ganglion cell axon bundle area in the retinas was quantified as the Tubulin β3-positive area. Laminin thickness at the innermost retina was quantified by manually measuring the thickness of the laminin-positive area at 100 µm from the optic disc on the retinal section. We did not measure ganglion cell axon thickness because it varied considerably by location.

Statistical analysis

Binary variables were compared by the χ^2 test or Fisher's exact test as appropriate. We created a 2×2 contingency table according to two categorical variables, OCT grade (0 or 1 vs 3 or 4) and DR stage (no DR, mild NPDR, or moderate NPDR vs severe NPDR or PDR), so that the expected value in each cell becomes greater than 5, which is an essential condition for the χ^2 test. Continuous variables were compared using the Mann-Whitney U-test. Mean color area ratios at different stages of DR were analyzed by the Kruskal-Wallis test followed by the multiple-comparison test, and mean changes in color area ratios over time were analyzed by the Wilcoxon signed-rank test. In all analyses, a p value less than 0.05 was considered to indicate statistical significance. Statistical analyses were performed using MedCalc software V.11.3 (MedCalc Software bvba, Mariakerke, Belgium).

RESULTS

In this retrospective longitudinal study, a total of 72 eyes of 72 patients with DM (all Japanese) were included. The baseline characteristics of the study population are presented in table 1.

Of the 72 eyes, 18 (25%) were previously treated with panretinal photocoagulation, and 16 (22%) had a previous history of diabetic macular edema.

The distributions of the grade and color area ratios on en face slab OCT images at baseline are presented in figure 2. In the analysis of the relationship between en face slab OCT grade and DR stage, OCT grade exhibited a statistically significant increase with advancing DR stage (p=0.018) (figure 2A). With regard to color distribution, the mean area ratio of red color significantly decreased (p=0.006) with advancing DR stage, whereas that of blue color showed a significant increase (p=0.003). There was no statistically significant change in the mean area ratio of green color (p=0.295) (figure 2B).

After a mean follow-up period of 4.6±1.5 years, both the stage of nephropathy and the stage of retinopathy significantly progressed, and the mean CRT and macular volume significantly decreased, whereas the mean hemoglobin A1c and logarithm of the minimal angle of resolution (logMAR) BCVA were comparable to values at baseline. During the follow-up period, 30 of 72 eyes (42%) underwent panretinal photocoagulation, and 9 (13%) had macular edema. The characteristics of the study eyes after follow-up are itemized in online supplementary

Table 1	Baseline characteristics
Eyes (n)	72
Age (years)	62.2±12.5
Gender, n (%)	
Male	46 (64)
Female	26 (36)
Type of diabetes, n (%)	
Type 1	3 (4)
Type 2	69 (96)
Duration of diabetes (years)	15.3±8.5
Duration of DR (years)	4.4±4.1
NGSP HbA1c (%)*	7.37±1.23
IFCC HbA1c (mmol/mol)*	57.0±13.4
Diabetic nephropathy,† n (%)	
Stage 1	6 (8)
Stage 2	27 (38)
Stage 3	11 (15)
Stage 4	5 (7)
Stage 5	1 (1)
Unknown	22 (31)
Lens status, n (%)	
Phakic	52 (72)
Intraocular lens	16 (22)
Unknown	4 (6)
DR, n (%)	
No DR	22 (31)
Mild NPDR	9 (13)
Moderate NPDR	13 (18)
Severe NPDR	8 (11)
PDR	17 (24)
Unknown	3 (4)
LogMAR BCVA	0.036±0.159
Central retinal thickness (µm)	260.9±35.4
Macular volume (mm³)	10.46±1.05
mGCIPL thickness (µm)	79.8±15.0
History of PRP, n(%)	18 (25)

Data are provided as mean±SD or n (%).
*n=53.
†Classified according to Classification of Diabetic Nephropathy 2014, published in *Clin Exp Nephrol (2015) 19:1–5*.
BCVA, best-corrected visual acuity; DR, diabetic retinopathy; HbA1c, hemoglobin A1c; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; logMAR, logarithm of the minimal angle of resolution; mGCIPL, macular ganglion cell complex–inner plexiform layer; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; PRP, panretinal photocoagulation.

table 1. In the analysis of 42 untreated eyes during the follow-up period, the distribution of OCT grades shifted to higher grades. The percentages of grades 0, 1, 2, and 3 were 28%, 42%, 26%, and 4%, respectively, at baseline and 15%, 46%, 26%, and 13% after follow-up (p<0.001, χ^2 test using a 2×2 contingency table (grade 0 or 1 vs 3 or four and at baseline vs after follow-up)) (figure 2C). The mean color area ratio was decreased in red (from
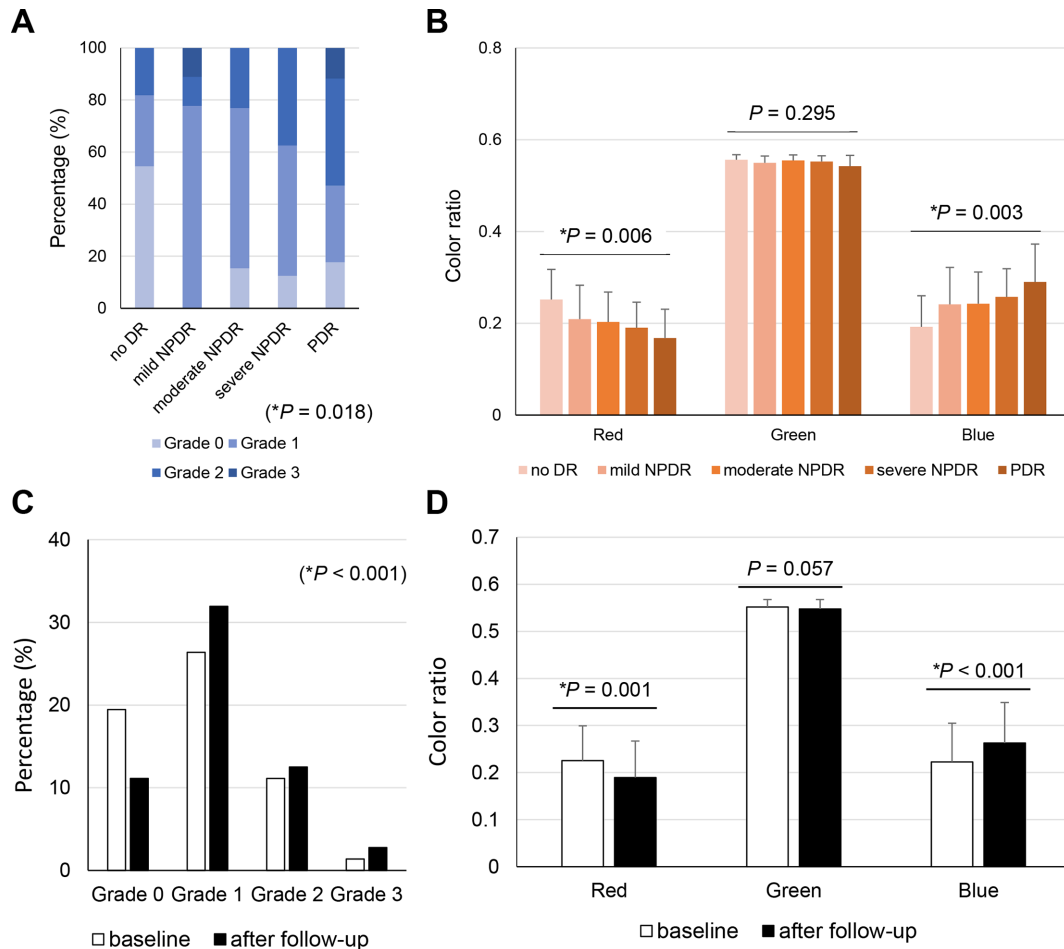
Figure 2 Distribution of the grade and color area ratio on en face slab OCT images at baseline and after more than 3 years of follow-up. (A) Distribution of en face slab OCT grades and DR stages. There was a significant increase in OCT grade with advancing DR stage (p=0.018). (B) Color area ratio on en face slab OCT image and DR stage. There was a significant decrease in the mean red color area ratio with advancing DR stage (p=0.006). Whereas the mean area ratio of green did not show a significant change (p=0.295), that of blue exhibited a significant increase with advancing DR stage (p=0.003). (C) The distribution of en face slab OCT grades showed a significant shift to higher grades (p<0.001). (D) There was a significant decrease in the mean color area ratio of red (p=0.001), a non-significant change in that of green (p=0.057), and a significant increase in that of blue (p<0.001). DR, diabetic retinopathy; NPDR; non-proliferative diabetic retinopathy; OCT, optical coherence tomography; PDR, proliferative diabetic retinopathy.

0.211±0.074 to 0.180±0.075, p=0.001), unchanged in green (from 0.550±0.017 to 0.544±0.024, p=0.057), and increased in blue (from 0.238±0.082 to 0.277±0.090, p<0.001) (figure 2D). A representative case in which en face slab OCT imaging successfully monitored the changes in the innermost layer of the diabetic retina over time is presented in figure 1E–H.

The association between color area ratio on en face slab OCT images and logMAR BCVA at baseline is presented in figure 3. There was a negative correlation between red color ratio and logMAR BCVA, no significant correlation between green color ratio and logMAR BCVA, and a positive correlation between blue color ratio and logMAR BCVA. After follow-up, all color ratios exhibited significant correlations with logMAR BCVA. The correlation was negative for red and green colors and positive for blue color (see online supplementary figure 1). Examination of the correlations between color ratio on en face

slab OCT images and macular thickness found no significant correlation for any color at any time point, except for green color after follow-up, which exhibited a weak positive correlation with CRT (see online supplementary figures 2 and 3). There was no significant correlation between color area ratio and mGCIPL thickness at baseline (see online supplementary figure 4). The mean logMAR BCVA was comparable among OCT grades at baseline, but a significant change was observed after follow-up: mean BCVA decreased (mean logMAR BCVA increased) with OCT grade progression. There were no significant differences in mean CRT among OCT grades at any time point (see online supplementary figure 5).

From a clinical point of view, it is important to assess the degree of neuronal cell loss, because neuronal cell loss directly leads to visual impairment. However, OCT evaluation of the inner retinal layer cannot distinguish neuronal from non-neuronal elements. Accordingly,
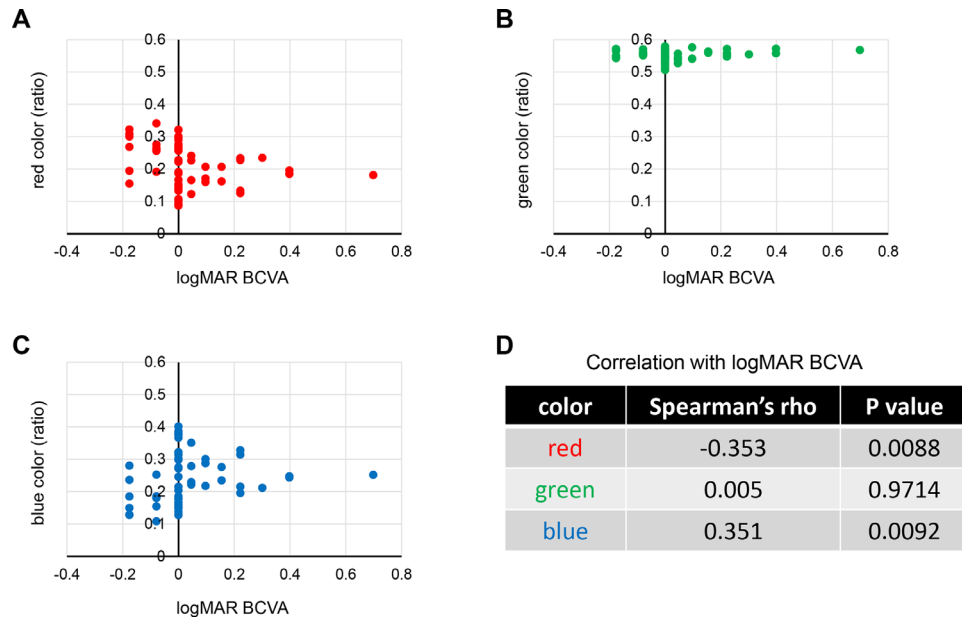
Figure 3 Color area ratio on en face slab optical coherence tomography images and visual acuity at baseline. (A–D) The color area ratio of red shows a significant negative correlation with logMAR BCVA. There were no significant correlations between the color ratio of green or blue and logMAR BCVA. BCVA, best-corrected visual acuity; logMAR, logarithm of the minimal angle of resolution.

to obtain insight into the pathogenesis represented by en face slab OCT imaging, we analyzed the retinas of non-obese hyperglycemic ($\beta Pdk1^{-/-}$) mice repeatedly backcrossed to the C57BL/6J genetic background. This congenic mouse exhibited minimal interindividual variation in body weight and blood glucose level, with coefficients of variation of <0.25, as presented in online supplementary figure 6. At the age of 20 weeks, diabetic $\beta Pdk1^{-/-}$ mice exhibited a significant attenuation of ganglion axon bundles immunolabeled with Tubulin β3 and thinner Laminin deposition at the innermost retinal layer compared with controls (figure 4; online supplementary figure 7). Whole-mount IHC exhibited no apparent differences between control and diabetic mice in CD31-labeled retinal endothelial cells, αSMA-labeled pericytes, and GFAP-labeled astrocytes. In addition, section IHC exhibited no significant alteration of immunoreactivity to other extracellular matrices, Fibronectin, and Type 4 collagen in diabetic mice (figure 4, online supplementary figure 8).

DISCUSSION

For the clinical application of en face slab OCT imaging, we developed a grading system in which the degree of retinal damage was assessed by the ratio of dark area to total area. This grading system has good intra-rater and inter-rater correlation (intraclass correlation coefficient, 0.847 and 0.818, respectively) and enables an intuitive understanding of the damage to the inner retina in clinical practice. As presented in figure 2A, our grading system worked well in that it could show the degree of reflectance change in the innermost retina with advancing DR stage, which was endorsed by our detailed

analyses using color distribution. DR progression has been assessed by ophthalmoscopy, fundus photography, and fluorescein angiography for a long time. With the advent of other modalities, such as OCT and OCT angiography, it became feasible to detect subtle changes in retinal thickness and the microvascular network in diabetic eyes. Those modalities provide additional information on DR status,[9] and the en face slab OCT imaging we exploited is a novel method.

There are several modalities to detect signs of retinal neurodegeneration in diabetic eyes.[2 12–14 17 29 30] Of those, retinal thickness measured by OCT and mfERG abnormalities can objectively monitor the progression of diabetic retinal neurodegeneration. Kim et al followed up type 2 diabetic patients for ≥4 years and evaluated the progression of retinal neurodegeneration by loss of thickness of mGCIPL measured using Cirrus HD-OCT.[17] Pinilla et al examined changes in retinal layer thickness by OCT in patients with type 1 DM who had no DR after 8 years of follow-up and found a significant difference in the thinning of the inner retinal layers compared with healthy controls.[31] Simó et al carried out a 2-year RCT to assess the efficacy of neuroprotective drug eye drops on retinal neurofunction in type 2 diabetic patients.[30] To test whether en face slab OCT imaging can assess temporal changes in the innermost retinal layer in diabetic eyes, we analyzed en face slab OCT image data from patients with DM who had been followed up with Cirrus HD-OCT for over 3 years. Our attempt turned out to be successful, as a statistically significant deterioration was observed in both assessments using OCT grading and color distribution analysis. We also showed that both CRT and mGCIPL thickness decreased after years of follow-up, indicating
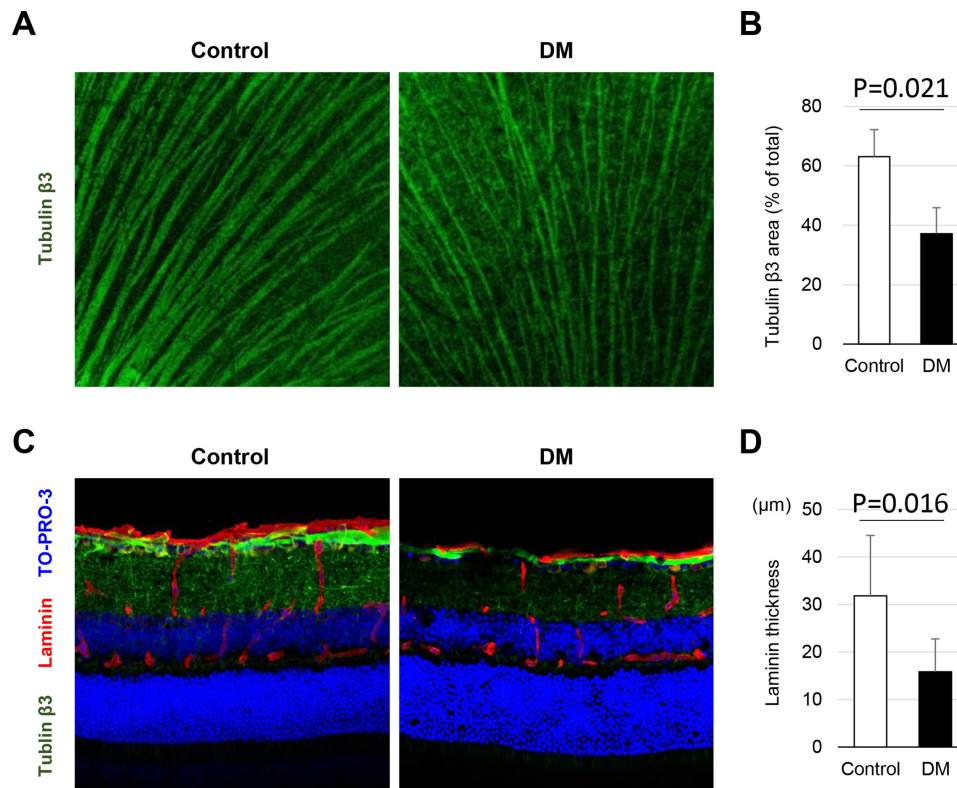
Figure 4 Pathological changes in the innermost retina in the diabetic model mouse. At the age of 20 weeks, there was a significant loss of ganglion axon bundles labeled with Tubulin β3 (A,B) and a significant decrease in Laminin thickness in the innermost retina (C,D) in diabetic β$Pdk1^{-/-}$ mice compared with control $Pdk1^{flox/flox}$ mice. DM, diabetes mellitus.

that retinal neurodegeneration progressed in our cohort. Taken together, the progression of diabetic retinal neurodegeneration could be monitored by en face slab OCT imaging, which employs changes in 2D optical properties in the innermost retina.

Central vision and retinal thickness are the two most important indexes for the assessment of vitreoretinal disorders. They have been used as main outcome measures in numerous RCTs.[32–34] Therefore, we tested the relationship between en face slab OCT images and these indexes. LogMAR BCVA showed a weak but significant negative correlation with the ratio of red to whole color on en face slab OCT images. This result shows that eyes with warm colors on en face slab OCT images are more likely to have good vision (note that smaller logMAR BCVA values indicate better Snellen or decimal BCVAs). On the other hand, CRT, which represents macular thickness, did not exhibit a clinically meaningful correlation with values obtained from en face slab OCT images. There are other OCT-based markers that correlate with BCVA, such as ellipsoid zone (EZ), external limiting membrane (ELM), and disorganization of retinal inner layers (DRIL).[35–37] EZ and ELM are hyperreflective lines at the outer retina, and both predominantly represent the condition of photoreceptors. Therefore, loss of EZ or disrupted ELM easily leads to decreased vision. However, structural damage to the outer retina usually occurs at advanced stages of DR.[35 36] DRIL strikingly displays irreversible changes in the inner retinal layers and appears to

be highly correlated with BCVA in current and resolved diabetic macular edema.[37] DRIL would probably be a robust marker of advanced retinal neurodegeneration because it is associated with increasing DR severity.[38] By contrast, en face slab OCT imaging can yield information on neuroretinal damage at early stages of DR. Our data show that 46% of eyes had grade 1 or 2 abnormalities on en face slab OCT images. In addition, the red color ratio produced by en face slab OCT imaging was not highly correlated with CRT or mGCIPL thickness. Taken together, en face slab OCT images could be associated with BCVA and provide novel information on retinal optical changes that reflect retinal neurodegeneration from an early stage of DR.

To gain insight into the characteristics of early pathological processes in the diabetic retina in which we think en face slab OCT imaging could detect subclinical changes, we used genetically modified non-obese hyperglycemic (β$Pdk1^{-/-}$) mice that we had developed.[26] β$Pdk1^{-/-}$ mice show a substantially high blood glucose level by 8 weeks of age due to a loss of pancreatic β cell mass without body weight gain. One of the advantages of β$Pdk1^{-/-}$ mice over other diabetic mouse models is that all the mice have the same known genetic background, and therefore, individual differences are expected to be smaller than those in other models, such as streptozotocin (STZ)-induced diabetic mice. At 20 weeks of age, β$Pdk1^{-/-}$ mice exhibited attenuation of ganglion axon bundles and reduction of Laminin deposition in the innermost

retinal layer compared with controls, whereas no overt changes were observed in retinal vessels, pericytes, and astrocytes. In reported mouse models of diabetes, the onset of retinal neurodegeneration is inconsistent.[39–43] In our diabetic mouse model, the phenotype of early-onset inner retinal morphological changes was reproducible, exhibited small individual differences, and appeared without apparent retinal vessel abnormalities (detailed phenotypical analyses are under way). Laminin is a major extracellular component of the innermost retina, which was confirmed by immunohistochemical analyses in the human eyes.[44] In the brain, astrocytic Laminin maintains the integrity of the blood–brain barrier partly by regulating pericyte differentiation.[45] As loss of pericytes and subsequent breakdown of the blood–retina barrier are one of the critical steps in the development of DR, the decreased Laminin thickness observed in our experiments may be an earlier event in the diabetic retina. From an optical point of view, both ganglion axon bundles and Laminin would be visualized as hyper-reflective lines on OCT sectional images and hyper-reflective areas on en face OCT images, because the difference in refractive index between fiber bundles and underlying cellular layer is sufficient to cause light scattering. In fact, Cuenca *et al* documented that the innermost retina was displayed as a broad hyper-reflective line on section OCT by comparing histological retinal structures using human donor eyes.[22] Accordingly, we think that the dark area observed on en face slab OCT images represents the loss of ganglion axon bundles or Laminin, or both. 'Structure–function' discordance is observed in some situations in ocular pathology, including glaucoma and optic neuritis. For instance, we previously reported that there was no correlation between visual field damage and mGCIPL and circumpapillary RNFL (cpRNFL) thickness in patients with anti-Aquaporin-4 antibody-negative optic neuritis but that there was a strong correlation between them in patients with anti-Aquaporin-4 antibody-positive optic neuritis, despite similar reduction in mGCIPL and cpRNFL thickness 6 months after the optic neuritis episode between the two.[46] This implies that the reduction in thickness is the sum of the reduction in neuronal and non-neuronal elements. The present animal data, in which axonal loss coincided with laminin volume reduction, corroborates this idea and claims the limitation of the structural change evaluation based on 'thickness', which is routinely conducted in current clinical settings. En face slab imaging may play a complementary role in the structural alteration that occurs in ocular pathologies and correlates with visual function.

This study has potential limitations, most of which stem from its retrospective design. Given that many ocular and systemic factors may affect the neuronal status of the innermost retina in diabetic eyes, the sample size was too small to definitely conclude that findings from en face slab OCT imaging are a robust marker of neurodegeneration. Media opacity and/or contour of the posterior retina caused by staphyloma or fundus tilt would affect the signal strength of the en face slab OCT image (we excluded such cases). It remains unclear whether the loss of ganglion axon bundles and Laminin observed in our diabetic mouse model truly represents the dark area observed on en face slab OCT images because of a technical problem: a reliable en face slab OCT imaging method has not been established in mice. Of course, findings from animal models do not precisely reflect human pathology.

In summary, en face slab OCT imaging can detect subtle structural changes in the innermost retina in diabetic eyes. Alterations of en face slab OCT images could be useful biomarkers for monitoring diabetic retinal neurodegeneration. Both the extracellular matrix and the neurons would be impaired from the early stages of diabetic retinal neurodegeneration.

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