Characterization of Retinal Vascular and Neural Damage in a Novel Model of Diabetic Retinopathy

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Submitted: December 19, 2014
Accepted: April 27, 2015

Diabetic retinopathy (DR) results from prolonged hyperglycemia and is characterized by significant changes in the vasculature and neural structure of the retina. Despite established screening programs and early treatment, DR remains an important cause of visual loss in working-age adults in developed countries.1 Clinically, DR has two principal stages.2,3 The first phase, nonproliferative DR (NPDR), is characterized by retinal microvascular changes such as pericyte loss, basement membrane thickening, formation of microaneurysms, and intraretinal microvascular abnormalities.4 The more serious proliferative DR (PDR) stage is characterized by the development of new blood vessels (neovascularisation) on the surface of the retina. This in turn leads to fibrovascular changes and tractional retinal detachment resulting in blindness.5 In addition to these vascular changes, neurodegeneration in the diabetic retina is indicated by loss of specific cell types and reduced retinal layer thickness.6 The mechanisms underlying development and progression of DR are incompletely understood. There is a need for a suitable animal model to understand both these mechanisms and the genetic factors contributing to DR.

Several mouse models have been described with retinal abnormalities during diabetes.7 However, none of these fully recapitulates the disease pathway seen in humans.8 Early NPDR features are seen in the spontaneously diabetic Akita (Ins2Aki-ta)9 and the streptozotocin (STZ)-induced C57BL/6j models.10 Neither model progressed to PDR, and only the Akita mouse showed significant loss of neural tissue.

Genetically modified mouse models can exhibit some of the retinal changes observed in DR.7 Mice overexpressing platelet derived growth factor (PDGF)-B exhibit early vascular phenotypes followed by some PDR characteristics in the vascular and neural retina.11,12 Development of NPDR lesions progressing to PDR was also noted in transgenic mice overexpressing insulin-like growth factor (IGF). However, neural damage to the retina was not described in these mice.13 Endothelial nitric oxide synthase (eNOS) knockout diabetic mice also developed several NPDR features, together with PDR lesions and neurodegeneration, as indicated by gliosis of Müller cells.14 Vascular endothelial growth factor (VEGF) transgenic mice models were reported to mimic most human DR features and have been extensively used to study sequelae of diabetes-induced eye disease. Some VEGF models show NPDR features, including severe damage to retinal vascular and neural structure, while others show slow progression with mild features and minimal or no neural damage.15–19 Although the VEGF model exhibits many of the features characteristic of human DR, it is limited by lack of hyperglycemia, presence of genetic modifications, and the direction of neovascularisation.18,19 While new retinal vessels invade the originally avascular vitreous in human DR, neovascularisation in VEGF models occurs in the opposite
A suitable animal model to investigate diabetic eye disease should closely mirror the clinical features of human DR. It is evident that the majority of animal models of DR demonstrate only early or severe changes of retinopathy, and to date there is no ideal animal model of DR. Here, we used the novel mouse resource “The Collaborative Cross” (CC) to develop a suitable mouse model of conditions observed in human DR. The CC is a genetic reference population established from eight diverse founder strains and captures a high degree of genetic diversity, with polymorphisms on average approximately every 200 bp. Each CC strain is unique, having a distinct mosaic genome pattern of alleles inherited from the eight founders, which encompass over 90% of common genetic diversity of the species. Therefore, the CC strains should harbor phenotypic diversity in any trait of interest. The aim of this study was to harness the power of the CC to establish a new mouse model having a range of hallmark features observed in human DR.

**Materials and Methods**

**Animals**

All experimental and animal handling activities were performed in accordance with the guidelines of institutional Animal Ethics Committee and ARVO statement for the Use of Animals in Ophthalmic and Vision Research. The principles, development, and initial characterization of the CC have been described. Mice were bred at the Animal Resources Center (Perth, WA, Australia) and were generously provided by Geniad Pty Ltd. (Perth, WA, Australia). Male and female mice were used at 8 to 10 weeks of age, housed under a 12-hour light/dark cycle and given a standard diet with free access to food and water. Diabetes was induced with a single dose (60–80 mg/kg) of alloxan and STZ (180–200 mg/kg; Sigma-Aldrich, Sydney, Australia) dissolved immediately prior to use in saline and used the novel mouse resource. Diabetes was induced with a single dose (60–80 mg/kg) of alloxan and STZ (180–200 mg/kg; Sigma-Aldrich, Sydney, Australia) dissolved immediately prior to use in saline and used the novel mouse resource.

**Retinal Isolation, Whole-Mount Preparation, and Analysis**

Freshly dissected retinas were fixed immediately in 4% paraformaldehyde (pH 7.3) for 5 minutes at room temperature followed by washing in PBS. Retinas were incubated overnight at 4°C with endothelial cell marker biotinylated *Griffonia (Bandeiraea) simplicifolia* Lectin I Isolectin-B4 (Isolectin-B4, 1:100 in PBS; Vector Laboratories, Burlingame, CA, USA), followed by 3 hours at 4°C in Cy3-Streptavidin (1:500 in PBS; GE Healthcare, Amersham, UK). Following incubation, samples were washed in PBS, mounted, and cover-slipped with Vectashield hard-set mounting medium (Vector Laboratories).

The overall retinal vasculature of labeled whole mounts was evaluated from the vitreous side of all diabetic and control retinas using fluorescent microscopy. The detailed structure of the vascular beds and lesions was analyzed by capturing representative areas in the Z-plane at 1- to 2-μm steps.

**Retinal Trypsin Digest Preparation and Analysis**

Whole eyes were processed to isolate the intact retinal microvasculature using 2.5% Trypsin (Invitrogen, Victoria, Australia) overnight at 37°C. Retinal vasculature was then mounted on a slide, stained using Periodic Acid Schiff’s (PAS) reagent, cover-slipped with glycerol, and stored at 4°C. Trypsin digest preparations were imaged using bright field microscopy; the number of acellular capillaries was counted in eight random fields and the numbers were normalized to the relative capillary density (number of acellular capillaries per millimeter squared of capillary area). The preparation was also screened for pericyte ghosts.

**Section Preparation and Analysis**

Enucleated eyes were fixed in 4% paraformaldehyde for 24 hours, placed in 70% ethanol overnight, followed by wax embedding. Paraffin sections (5 μm) were collected and stained with (1) hematoxylin and eosin (H&E), (2) glial fibrillary acid protein (GFAP, 1:3000; Dako, City, Denmark), and (3) Isolectin-B4 (Vector Laboratories) at 4°C overnight, Cy3-Streptavidin (GE Healthcare) and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclei staining (Sigma-Aldrich) followed by bright field or fluorescent microscopy.

Sections stained with H&E were used for evaluating the number of ganglion cells in the ganglion cell layer (GCL). Ganglion cells were counted in approximately 500- to 700-μm linear distance in central, mid, and peripheral retinal regions. Expression of GFAP was visualized in Müller cell projections spanning layers situated toward the ONL of the GCL, from central, mid, and peripheral retinal regions. Hematoxylin and eosin– and GFAP-stained sections were also scanned for changes in retinal layers. Isolectin-B4– and Cy3-Streptavidin–stained retinal sections were scanned for intraretinal neurovascular lesions from the inner limiting membrane toward the outer retina. Similarly, blood vessels protruding through the inner limiting membrane into the vitreous were considered for preretinal neovascularization. At least six sections per retina were assessed.

**Microscopy**

Retinal whole-mounts were analyzed using an Olympus IX71 fluorescent microscope with a digital camera Olympus DP70 linked to computer running image analysis software DP Controller (Olympus, Tokyo, Japan). Detailed structure of retinal whole-mounts, trypsin digest preparations, retinal sections stained with isolecitin-B4 and Cy3-Streptavidin were...
A Novel Mouse Model of DR

**FIGURE 1.** Characterization of the retinal vasculature in nondiabetic control (A) and diabetic FOT (B-E) mice. Freshly dissected retinas were fixed immediately then stained with an endothelial cell marker and visualized. Nondiabetic mice had an organized retinal vascular branching pattern (A). After 7d of diabetes, the vascular distribution in retinas of FOT mice was dense and disorganized (B) and this deteriorated by 21d after diabetes induction (C). Formation of microaneurysms in retinal capillary walls (D) and intraretinal microvascular abnormalities formation by repeated folding of capillaries (E) were observed at 21d of diabetes. Scale bars: 50 μm (A-E).
Microarray

Whole retinas were collected in Trizol and purified using the manufactures instructions (Invitrogen, Grand Island, NY, USA). Each sample consisted of three retinas in groups of either diabetic or control mice; three samples of each group were then run on the array. RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and quality checked with the Total RNA Nano 6000 Assay Kit (Agilent Technologies, Victoria, Australia) and the Agilent Bioanalyser. All RNA integrity (RIN) values were 7 or higher. Synthesis of cRNA was performed using an Illumina Total-PrepTM RNA Amplification Kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. A total of 750 ng labeled cRNA was suspended in 5 µL RNase free water and hybridized to a BeadChip Array MouseRef-8 V2 (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions, followed by washing and staining with Streptavidin Cy-3 (GE Heathcare, Amersham, UK). The BeadChip was scanned with Bead Array Reader (Illumina) using Illumina Bead Studio software (Illumina).

The data were further analyzed in the ‘R’ statistical environment. Transcripts that had a detection $P$ value less than 0.05 in greater than 10% of the sample set were obtained using the LUMI R module. Following this, negative gene expression values (which do not have any biological meaning) were removed. Other experimental-, biological-, and machine-related variations were then eliminated by transforming data sets to Log2 scale values and normalizing using a Robust Spline Normalization (RSN) algorithm. In order to ensure the accuracy of the normalizing process, diagnostic plots were generated. Sample replicates clustering together and aligning to the same median value indicates correct normalization. Following the above procedure, Principle Component Analysis (PCA) was applied to the normalized microarray data sets. Samples were subject to differential expression analysis using the empirical Bayesian technique described in the LIMMA R package and a False Discovery Rate (FDR) correction was applied to the $P$ values. Genes were considered differentially expressed if they had Benjamini-corrected FDR $P$ values smaller than 0.05 together with a greater than 0.5 log 2-fold change (either positive or negative).

Statistical Analysis

All morphometric data were analyzed using two-tailed Student’s $t$-test or ANOVA where appropriate, and $P$ less than 0.05 was considered as a significant threshold.

RESULTS

Mice from a total of 64 strains from our CC colony$^{22}$ were tested. All mice readily developed diabetes after administration of alloxan or STZ. Diabetic mice from all strains were screened for DR lesions. Flat-mount retinal preparations stained for blood vessels were first evaluated by visualizing the overall retinal vasculature in each retinal quadrant. The detailed structure of the vascular beds was visualized by capturing representative areas in Z-plane using an inverted fluorescence microscopic system. During this analysis, the vasculature was carefully screened for the presence of any microvascular lesions such as microaneurysms. At least 10 mice per strain were examined as described. From this screen, the most

FIGURE 2. Retinal vasculature in control (A) and STZ-diabetic FOT (B, C) mice. Freshly dissected retinas were fixed immediately then stained with an endothelial cell marker and visualized. Nondiabetic mice had an organized retinal vasculature (A). After 7d of STZ-diabetes, the vascular distribution was disorganized (B), which deteriorated by 21d of STZ-diabetes (C). Scale bar: 50 µm (A–E).
promising strain (FOT_FB, referred to hereafter as FOT) was selected for further studies.

Despite their induced diabetes, alloxan or STZ-treated FOT mice showed no weight loss over the 21-day experimental period (not shown). No obvious gross anatomical abnormalities were observed and there was no significant difference in whole-eye weight (mg) between control and diabetic mice, at either 7d (control versus 7d diabetic: 22.6 ± 1.1 and 21.9 ± 0.4; P > 0.05) or 21d (control versus 21d diabetic: 22.6 ± 1.1 and 22.0 ± 0.5; P > 0.05).

**Early Vascular Lesions and Retinal Vascular Proliferation**

Control retinas showed a clear organized branching pattern of blood vessels (Fig. 1A). In contrast, a disorganized, dense distribution of capillaries was prominent in the peripheral retina as early as 7d after diabetes induction with alloxan (Fig. 1B). By 21d of diabetes there was a dense, convoluted retinal capillary network in both peripheral and mid retinal regions (Fig. 1C). This vascular distribution did not favor any specific retinal quadrant.

Vascular lesions were also noted at 21d (Figs. 1D, 1E). Microaneurysms appeared in the middle capillary bed, as evidenced by out-pouching or sac-like dilations of the retinal capillary walls (Fig. 1D). Repeated overlapping of retinal capillaries within the mid or/and lower capillary beds occasionally formed lesions resembling intraretinal microvascular abnormalities (Fig. 1E). These lesions were smaller when formed by capillary recruitment only from the middle capillary bed compared with those formed from both the middle and lower beds. No vascular lesions were observed at the earlier stage (7d) of diabetes.

In order to demonstrate that these lesions were due to hyperglycemia of diabetes, rather than to a putative, previously unknown side effect of alloxan, we also tested FOT mice rendered diabetic by STZ treatment. Streptozotocin and alloxan both induce diabetes by destruction of the insulin-producing beta cells, but they operate via different biochemical pathways.24 As expected, FOT mice rendered diabetic by STZ also showed neovascularization at 7d (Fig. 2B), 21d (Fig. 2C) compared to controls (Fig. 2A).
FIGURE 4. Retinal microvascular abnormalities in the diabetic FOT retina. Whole eyes were processed to isolate the intact retinal microvasculature using trypsin. The retinal vasculature was then mounted on a slide, stained with Periodic Acid Schiff’s, and analyzed using bright field microscopy. Normal retinal vasculature is seen in control (A). Retinal sections from diabetic FOT mice are shown with examples of acellular capillaries within close proximity (arrowheads [B]); apparently migrating pericytes (arrow [C]); and pericyte ghost in 7d diabetics (asterisk [D]). Numbers of acellular capillaries are shown (E) with mean ± SEM. *P < 0.001 control versus 7d diabetic, +P < 0.001 control versus 21d diabetic, and *P < 0.05 7d versus 21d diabetic. Scale bars: 50 μm (A–C), 10 μm (D).

Retinal vascular proliferation and location of lesions were studied in further detail in mice treated with alloxan (Figs. 3A, 3C, 3E). In retinal sections from control mice, the retinal capillaries were confined to the outer margin of the inner nuclear layer (INL; Fig. 3B). At 7d of diabetes, there were more retinal capillaries in the mid and deep capillary beds but these were limited to the outer margin of the INL (not shown). By 21d, the capillaries within retinal layers were dilated and formed convoluted intraretinal microvascular abnormalities-like structures (Fig. 3D). Occasionally, abnormal blood vessels penetrated toward the ONL, disrupting the outer retina (not shown). Periretinal neovascular tufts were also present, appearing as disorganized, clustered vessels growing into the normally avascular vitreous cavity (Fig. 3F).

Increased Acellular Capillaries and Presence of Pericyte Ghosts

To assess damage in the retinal vasculature, the presence of acellular capillaries and pericyte ghosts was analyzed in retinal trypsin digest preparations of both control and diabetic mice (Figs. 4A–D). Diabetes significantly increased the formation of acellular capillaries. At 21d, acellular capillaries were observed within close proximity of each other (Fig. 4B). There was a 2.3-fold increase in acellular capillaries in the 7d diabetic mice, and a further 1.3-fold increase in the 21d group (P < 0.001 versus controls; P < 0.05 versus 7d; Fig. 4E). Both 7d and 21d diabetic mice had migrating pericytes (Fig. 4C). Pericyte ghosts were occasionally observed in 7d diabetic (Fig. 3D) but not in 21d diabetic mice or controls.

Macroglial Activation

Müller cell gliosis is a distinct feature of human DR.²⁵ Retinal sections were stained with anti-GFAP antibody. In control retinas, GFAP immunoreactivity was limited to astrocytes and the endfeet of Müller cells, located in the nerve fiber layer and the GCL (Fig. 5A). Occasional radial Müller cell projections were activated, extending a short distance within the inner plexiform layer (IPL). Müller glial cell activation was observed in the peripheral retina of 7d diabetic mice (Fig. 5B) and was more severe at 21d (Fig. 5C). It was present in the peripheral, mid, and occasionally in the central retinal regions.

Neurodegeneration of the Retina

Loss of neural cells accompanying thinning and alterations of retinal layers was recently reported in human DR.²⁶ Neurodegeneration was assessed in Figs. 6A–F in diabetic FOT mice (Figs. 6A, 6D). There was evidence of loss of retinal ganglion cells (RGCs) in both 7d (Fig. 6B) and 21d diabetic mice (Figs. 6C, 6F). Retinal ganglion cells were more packed in controls (Fig. 6A) than in 7d diabetic mice (Fig. 6B). The 7d midretinal region (Fig. 6E) appeared more like the control midretina (Fig. 6D). At 21d, ganglion cell loss was evident and the distance between cells increased in both the peripheral and midretinal regions (Figs. 6C, 6F).

There was a significant reduction in the ganglion cell number in the periphery of 7d and 21d diabetic retinas (Fig. 6G; P < 0.05 and P < 0.001, respectively). Ganglion cells were also reduced in the mid retinal region of 21d diabetic mice (P < 0.05), and this reduction became worse with duration of diabetes, with a significant difference between the 7d and 21d time points (P < 0.05). However, there were no significant differences in the central retina areas between any groups.

The retinal layers situated toward the ONL, in both control (Figs. 6A, 6D) and 7d diabetic mice (Figs. 6B, 6E) appeared normal. In the midretinal regions of 21d diabetic mice, dilated blood vessels were observed that projected from the nerve fiber layer into the preretinal cavity, showing a congested appearance possibly representing venous dilation or beading (Fig. 6F). By 21d, diabetic mice had disruption to the photoreceptor layer with the ONL penetrating toward the retinal pigment epithelium (Fig. 6G). No significant difference was observed in the thickness of either the INL (Fig. 6J) or the ONL (Fig. 6K).

Differential Expression of Retinal Genes

Microarray analysis of 21d diabetic retinas showed changes in expression in 46 genes compared with controls. Expression was increased in 45 genes (+1.0 to +7.7 log-fold) and decreased in 1 gene (−1.42 log-fold). Among these, expression of several important genes such as Tfr, Rgr, Rhp1, and Rpe65 more than
doubled (Table). The one gene that was downregulated was anonymous (Table).

**DISCUSSION**

Several mouse models of DR have been reported, but none have been described that exhibit the range of features seen in human DR. Most of the models harbored genetic modifications designed to artificially over- or underexpress particular genes. Our approach was to identify a mouse strain that would develop retinopathy lesions in response to diabetes, and would do so via the interaction of common genetic polymorphisms, just as humans do. The CC 21,22 was ideal for this purpose as it maximizes genetic variation within a panel of inbred mouse strains.23 We found one strain, FOT, which showed profound changes in both retinal vasculature and neural structure as early as 7d from the onset of diabetes. Diabetic FOT mice developed a wide range of lesions similar to those seen in human DR. These included pericyte ghosts, increased acellular capillaries, microaneurysms, intraretinal microvascular abnormalities, vascular proliferation, Müller cell gliosis, ganglion cell loss, and disruption of the neural retina.

Retinal homeostasis is maintained by the proper function of the blood–retinal barrier. Pericytes are important in maintaining this barrier. The loss of these cells can have a significant impact on the retinal vasculature, and their loss is often accompanied by endothelial cell degeneration, leading to acellular capillaries. Hence, degeneration of pericytes and
blood flow is an early consequence of diabetes and can bring diabetic FOT mice showed dilated capillaries within the retina, months of diabetes, but no preretinal neovascular signs were increased with duration of diabetes, indicating an intraretinal which eventually leads to severe retinal changes.

Pericyte loss has been reported in humans and in some animal models, but infrequently detected in mice due to the small size of the mouse retinal capillaries. The number of acellular capillaries increases with diabetes in humans and rodents and was also evident in the FOT model. First reported migrating pericytes in diabetic eyes. The signs of migrating pericytes in FOT retina (Fig. 3C) indicated ongoing vascular damage in these mice. This suggests that early retinal vascular degeneration can contribute to ischemia followed by hypoxia, which eventually leads to severe retinal changes.

The formation of new blood vessels within the retina increased with duration of diabetes, indicating an intraretinal neovascularization rather than preretinal neovascularization. Similar findings have been reported in the Ins2Akita mouse at 9 months of diabetes, but no preretinal neovascular signs were observed in this model. Furthermore, retinal sections of diabetic FOT mice showed dilated capillaries within the retina, suggesting fluctuation of retinal blood flow. Increased retinal blood flow is an early consequence of diabetes and can bring about capillary dilation.

The newly formed blood vessels on the retinal surface that penetrate into the vitreous possibly do so in response to an hypoxic environment, and neovascularization may be initiated by interactions between ganglion cells and surrounding cells. Models of oxygen-induced retinopathy demonstrate preretinal neovascular abnormalities such as neovascular tuft formation. Studies on such models show the recruitment of ganglion cells leading to the formation of these abnormalities.

Reduced RGC numbers have been reported in diabetic subjects. The Ins2Akita model showed loss of RGCs from the peripheral retina, but not in the central retina after 3 months of diabetes. In the FOT mice, ganglion cell losses from both the mid- and peripheral retinal regions were seen as early as 5 weeks after the onset of diabetes.

RNA was isolated from the retinas of FOT mice that either had or had not been treated over a period of 4 weeks with (+)-alloxan and subjected to microarray analysis. Information is shown about genes with expression that was altered in the diabetic mouse compared with the control. Upregulated genes (≥2.0-fold change) and downregulated genes (≥1.0-fold change) are provided.
the CC was developed to assist in identifying genes mediating traits that can only be assessed in vivo,46 a potential that is now being realized.46 Most of the CC mice we tested did not display DR, suggesting the involvement of multiple DR susceptibility genes. Identification of these genes in our novel model will provide targets for new treatments of DR.

Acknowledgments

The authors thank Fred Chan (University of Western Australia) for advice and discussion of the manuscript. They also thank Ben Ezzy, Kylee Parentich, Andrew Wallace, and Glynn Manship for providing animal care services, and Terry Cop (Royal Perth Hospital, Perth, WA, Australia) for processing histology samples. The authors acknowledge the scientific and technical assistance of the Centre for Microscopy, Characterisation and Analysis of the University of Western Australia.

Supported by grants from the Diabetes Research Foundation of Western Australia (Perth, WA, Australia), Discovery Project 11010206 from the Australian Research Council (Canberra, ACT, Australia), and Program Grant 1037321 from the National Health and Medical Research Council of Australia (Canberra, ACT, Australia).

Disclosure: L.Y. Weerasekera, None; L.A. Balmer, None; R. Ram, None; G. Morahan, None

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