Histologic Characterization of Retina Neuroglia Modifications in Diabetic Zucker Diabetic Fatty Rats

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PURPOSE. The purpose of this study was to characterize retinal degenerative morphologic modifications in Zucker Diabetic Fatty (ZDF) rats, a genetic model of type 2 diabetes, by histologic and immunohistochemical evaluation.

METHODS. Male lean (C/; n = 10) and ZDF (fa/fa; n = 20) rats were used. At 24 weeks of age, body weights and blood glucose levels were determined. Eyes were removed and processed for paraffin wax embedding. Sections through the optic disc were stained for hematoxylin and eosin or immunostained for TUNEL, advanced glycation end products (AGEs), glial fibrillary acidic protein (GFAP), glutamate/aspartate transporter (GLAST), isocitrate B4, recoverin, retinal pigment epithelium-specific 65-kDa protein, rhodopsin, vimentin, and zonula occludens protein 1. Retinal morphometry, cell counts, glial activation degree and immunoreactivity of AGEs and GLAST were also determined.

RESULTS. ZDF rats were observed to be diabetic from week 9 and by week 24. These animals showed retinal morphologic degenerative changes, increased neuroretinal thickness, and decreased number of nuclei. Glial cells activation with massive GFAP upregulation was present. Cellular morphologic modifications were also observed. GLAST immunofluorescence was decreased, whereas AGEs were increased in comparison with lean rats.

CONCLUSIONS. Spontaneous development of diabetes in ZDF rats results in neuroglia morphologic degenerative changes at 24 weeks of age. This animal model may be useful to understand the pathogenesis of diabetic retinopathy and to screen neuroprotective drugs in diabetes.

Keywords: Zucker diabetic fatty (ZDF) rats, diabetic retinopathy, rodent models, neuroglia

A t present the global population with diabetes mellitus is 415 million, and it is projected that the number of diabetic patients will reach 642 million by 2040 (http://www.dia betesatlas.org; assessed January 2017). Diabetes can be generally divided into two types: type 1 (insulin dependent) and type 2 (insulin independent), of which hyperglycemia is a common feature. The ophthalmic complications of hyperglycemia are most profound in the retina accounting for most visual loss in diabetics, and diabetic retinopathy (DR) is the leading cause of blindness among working-aged adults around the world.1,2

DR is understood as a microvasculopathy due to a loss of microcapillaries in early stages and an increase in retinal vascular permeability resulting in the leakage of plasma into the surrounding tissues. There are reports suggesting that hyperglycemia and extracellular reactive oxygen species are toxic to endothelial cells, pericytes, neurons, and glial cells, resulting in cellular alterations in early DR.3,4 Neuroglial abnormalities are still being explored to determine their clinical significance. Furthermore, there is accumulating evidence that low-grade inflammation underlies the vascular complications of DR.3,6

Human studies have provided some information on the development of retinal complications associated with diabetes; however, the mechanisms underlying the development of DR have yet to be fully elucidated. The use of animal models of DR enable us to have a more comprehensive understanding of the pathophysiology at the molecular and cellular level in a controlled manner and also fulfill the need for drug screening tools. Many animal models of DR have been developed.7 Although most animal models are well characterized concerning retinal microvasculature modifications, few have been studied in detail about the retinal degenerative modifications associated with hyperglycemia.7

Diabetic rat models can be induced by diet, pharmacologic or toxic agents, or by animals carrying an endogenous genetic mutation. A number of rats with spontaneous onset of diabetes have been identified.7 The Zucker Diabetic Fatty (ZDF) rats are a genetic model of type 2 diabetes, predominantly investigated for the characterization of diabetic nephropathy,8 whereas retinal modifications are as yet incompletely described. ZDF rats carry an inherited mutation of the fa/fa gene, which leads to a phenotype of insulin resistance and results in impairment of glucose tolerance. Excessive body weight gain was observed in male ZDF rats in the first 6 months of life, but the weight decreases to a level similar to the lean controls afterward.9 Hyperglycemia starts at 6 to 7 weeks of age and is high
throughout their life. Retinal functional analysis of ZDF rats was recently described. However, profound cellular and molecular studies of the retina have not yet been performed in those animals. In this study, we characterized retinal degenerative morphologic modifications in ZDF rats by histologic and immunohistochemical evaluation.

**METHODS**

**Animals**

The use of animals in this study was in accordance with the recommendations of ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This study was approved by the Animal Research and Welfare Ethics Committee of the University of Nottingham Medical School. Male ZDF (fa/fa; n = 20) and lean counterparts (C/); n = 10) rats (Charles River, Waltham, MA, USA) were used in this study. Animals were 6 weeks of age on arrival and were housed in pairs to week 11 and singly thereafter. Animals were maintained under standard laboratory conditions with free access to food (Purina 5008 diet; Charles River) and water. Body weights were determined at least weekly, and plasma glucose and glycated hemoglobin (HbA1c) were monitored at various times. At 24 weeks of age, the animals were euthanized, and the eyeballs were removed.

**Determination of Plasma Glucose and Blood HbA1c**

Following a 4-hour fast, blood was collected from the tail view in lithium heparin tubes, plasma separated by centrifugation, and stored at −20°C until analysis. Plasma glucose concentration was determined using a clinical glucose assay reagent (Thermo Electron Infinity stable reagent TR15421; Thermo-Fisher Scientific, Paisley, UK). All determinations were performed in duplicate. A glucose standard (Sigma-G6918L, 5.55 mM; Sigma-Aldrich, St. Louis, MO, USA) was included on all assay plates to confirm correct assay performance. Fasted plasma samples were diluted five times in saline. Standards and unknowns were pipetted into 96-well plates (FX9200 flat bottomed; Alpha Laboratories, Eastleigh, UK) as 20-µL additions. The reaction was started by adding 250 µL assay reagent followed by incubation at 37°C for 10 to 15 minutes, after which optical density at 340 and 400 nm (correcting wavelength) was determined using a SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA, USA). The optical density change was then calculated by subtraction.

HbA1c levels in whole blood, collected into lithium heparin tubes, were determined with the Cobas c111 analyzer using the PreciControl HbA1c norm (Roche Diagnostics Limited) and PreciControl HbA1C path (Roche Diagnostics Limited) were used for blood HbA1c and returned acceptable values.

**Tissue Processing and Staining**

Eyes were fixed at least 24 hours in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and processed in an automatic tissue processor (Leica ASP500; Leica Microsystems, Wetzlar, Germany). One paraffin block from each eye was made, and vertical serial 5-µm-thick sections through the optic disc were obtained in a rotatory microtome (Microm HM540E; Microm International GmbH, Walldorf, Germany).

Ocular sections for hematoxylin and eosin (HE) staining were de-waxed in xylene, rehydrated in a series of descending alcohols, rinsed in deionized distilled water, and stained with HE (Merck KGaA, Darmstadt, Germany). Stained sections were dehydrated in a series of ascending alcohols, cleared in xylene, mounted, and coverslipped.

**Morphometry and Cell Counts**

The retinal parenchyma in HE-stained sections were analyzed by measuring the distance between the outer and inner limiting membranes (OLM and ILM); the thickness of the outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), and inner plexiform layer (IPL); and total retinal thickness in these sections were also measured. Two photographs were taken per section at 300 µm either side of the optic disc (central retina), with a magnification of ×40. Image J software version 1.49 (http://imagej.nih.gov/ij/; National Institutes of Health, Bethesda, MD, USA) was used to quantify retinal layer thickness. The number of cell nuclei per stack in the INL and ONL and the number of ectopic nuclei of HE-stained sections were manually counted from the captured images. The analysis of morphometry and cell nuclei number were conducted in the same retinal location and at the same magnification.

**Immunohistochemistry**

Ocular sections for immunohistochemistry were randomly selected from different animals (Table 1), de-waxed in xylene, dehydrated as described above, and rinsed in PBS (Gibco, Invitrogen, Paisley, UK). For antigen retrieval, sections were incubated in 0.01% Trypsin (Sigma-Aldrich, Steinheim, Germany) in PBS, for 15 minutes at 37°C. Then, TUNEL staining was performed with an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany), or immunostaining for the phenotype-specific markers (Table 1) advanced glycation end products (AGEs), glial fibrillary acidic protein (GFAP), glutamate/aspartate transporter (GLAST), isoleucin B4 (Ib4), recoverin (Rec), retinal pigment epithelium-specific 65-kDa protein (RPE65), rhodopsin (Rho), vimentin (Vim), and zonula occludens protein 1 (ZO1) was performed. Specific combinations of antibodies were diluted in PBS containing 0.5% Triton X-100 (Sigma-Aldrich) and incubated overnight at 4°C. The next day, the sections were washed in PBS. Thereafter, the corresponding species-specific secondary antibodies conjugated to Alexa Fluor 488 and/or 568 (green and red; ThermoFisher Scientific, Rockford, IL, USA) were applied at a 1:200 dilution. Nuclei were stained with Hoechst blue (1:500; Sigma-Aldrich). Finally, sections were washed in PBS, mounted in aqueous-based fluorescent mounting medium (Fluormount-G; SouthernBiotech, Birmingham, AL, USA), and coverslipped.

Control samples in which the terminal transferase or the primary antibodies were omitted were processed in parallel, and no immunoreactivity was found in any case. Samples were analyzed with a Leica Leitz DMRB light microscope (Leica Microsystems, Wetzlar, Germany) equipped for epifluorescence, and images were acquired with a Retiga 3000 CCD monochrome camera (QImaging, Surrey, Canada). Brightness and contrast were minimally adjusted, and final figures were composed with Pixelmator 3.4 Twist (Apple, Cupertino, CA, USA).

The number of TUNEL-positive elements was manually counted from two photographs per section, at 300 µm either
side of the optic disc (central retina), with a magnification of ×40. To evaluate the degree of glial activation, a scoring system based on extent of GFAP staining was used. Two minimum of three sections per animal was examined at ×20 magnification, and scores were assigned for central retina regions (at 300 μm either side of the optic disc) according to a five-point scoring system. The scoring system was as follows: Müller cell endfeet region/GCL only (score 1); Müller cell endfeet region/GCL plus a few proximal processes (score 2); Müller cell endfeet plus processes throughout with some in the ONL (score 3); Müller cell endfeet plus processes throughout with some in the ONL (score 4); Müller cell endfeet plus lots of processes from GCL to outer margin of ONL (score 5).

Immunoreactivity semiquantification was measured in ocular sections as previously described in the literature. Two-channel micrographs were analyzed using ImageJ software version 1.49 (National Institutes of Health). The micrographs were then split into different channels, and each channel was thresholded. To subtract background in the red channel (corresponding to the protein of interest), negative controls were used to set the threshold value, and the same value was used in all micrographs being analyzed from the same experiment at the same time. The threshold for the blue channel, corresponding to the area of nuclei, was set according to the area stained. Mean gray value was measured redirecting the measurement to the corresponding channel. The results, in arbitrary units, were the ratio of the mean gray value for the red channel to that obtained from the blue channel.

### Statistical Analysis

All data were collected in a database created in Excel (Microsoft Office Excel 2013; Microsoft Corporation, Redmond, WA, USA) and subsequently analyzed by Student’s t-test for one-tailed distribution and two-sample equal variance (homoscedastic).

#### RESULTS

### Body Weight, Plasma Glucose, and Blood HbA1c

On arrival, the body weight of the lean and ZDF rats was 192 ± 4.2 and 237 ± 5.4 g, respectively. Body weights initially rose more rapidly in the ZDF rats compared with the lean, but at the end of the experiment (24 weeks of age), the body weights were not significantly different between lean (401 ± 6.4 g) and ZDF (391 ± 4.7 g) rats. At 7 weeks of age, the blood glucose levels (4-hour fast) of the lean and ZDF rats were similar at 8.39 ± 0.24 and 9.67 ± 0.30 mM, respectively. The blood glucose levels of the lean animals remained essentially unchanged during the experiment, whereas the blood glucose levels of the ZDF rats rose to 18.5 ± 1.18, 26.12 ± 0.8, and 33.59 ± 0.6 mM at 9, 11, and 24 weeks, respectively. Thus, the ZDF animals were clearly diabetic from week 9 onward. At 24 weeks of age, the HbA1c values were 4.58 ± 0.05% and 10.05 ± 0.12% in the lean and ZDF rats, respectively (Table 2). The metabolic data presented above is the mean ± SEM.

### Retinal General Morphology, Morphometry, and Cell Counts

To evaluate general morphology of the retina tissue in lean and ZDF rats, HE staining was performed in ocular sections. In lean and ZDF animals, general morphologic organization of the retina was normal in appearance. Retinas showed the typical highly organized layered structure with cells regularly arranged (Fig. 1A). Photoreceptor outer and inner segments (ISs) were easily recognized, as well as the ONL, INL, and GCL layers. In ZDF rats, enclosed retinal regions showed initial morphologic degenerative changes, such as ONL and INL subtle misorganization and ectopic nuclei located outside the OLM in the photoreceptor IS area (Fig. 1B, arrowheads). Some ocular regions showed separation between the neuroretina and RPE that represent histologic artifacts (Figs. 1A, 1B, asterisks).

<table>
<thead>
<tr>
<th>Molecular Marker</th>
<th>Antibody</th>
<th>Source</th>
<th>Working Dilution</th>
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<th>ZDF</th>
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AGEs, advanced glycation end products; GFAP, glial fibrillary acidic protein; GLAST (EAAT1), glutamate/aspartate transporter; IB4, isoelectin B4 from Griffonia simplicifolia; Rec, recoverin; RPE65, retinal pigment epithelium-specific 65 KDa protein; Rho, rhodopsin; TUNEL, TdT-mediated dUTP nick end labeling; Vim, vimentin; ZO1, zonula occludens protein 1.

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**Table 1.** Phenotype-Specific Markers and Number of Animals (n) Used on Each Study

**Table 2.** Body Weight, Plasma Glucose, and Blood HbA1c Values of Lean and ZDF Rats

<table>
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<th>Body Weight (g)</th>
<th>Plasma Glucose (mM)</th>
<th>HbA1c (%)</th>
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<tr>
<td>Lean</td>
<td>6 wk</td>
<td>24 wk</td>
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<tr>
<td>192 ± 4.2</td>
<td>401 ± 6.4</td>
<td>8.39 ± 0.24</td>
</tr>
<tr>
<td>ZDF</td>
<td>237 ± 5.4*</td>
<td>391 ± 4.7</td>
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Data are expressed as mean ± SEM.
* P < 0.01 comparing lean and ZDF rats.
To determine whether there is a significant change in retina thickness and/or loss of cells, several measurements were performed in lean and ZDF rats retina samples. Measurements of retinal thickness and counting of cell nuclei per stack in lean and ZDF rats are shown in Figures 1C and 1D. Total central retina (measured from the ILM to the OLM) and OPL thicknesses were significantly increased in ZDF rats in comparison with lean rats. Thickening of the ONL, INL, and IPL was observed in ZDF animals. (D) Number of nuclei per stack in the ONL and INL was significantly decreased in ZDF in comparison with lean animals. HE staining (A, B). Separation between the neuroretina and RPE are histologic artifacts (A, B, asterisks). Lean (n = 7) and ZDF (n = 11) (C, D). Ch, choroid; GCL, ganglion cell layer. *P < 0.05 (C, D). Scale bar denotes 25 μm.

Immunohistochemistry Characterization

Cell Death. To determine whether there is an increase in cellular death rate, TUNEL staining for detecting DNA fragmentation that results from apoptotic signaling cascades was performed. In control sections, no immunoreactivity was found in any case (Fig. 2A). In both lean and ZDF animals (Figs. 2B, 2C), occasionally TUNEL-positive cells could be seen in the ONL, INL, and GCL. Only ZDF animals show TUNEL-positive ectopic nuclei located in the photoreceptors IS area (Fig. 2C, arrowheads). The total number of TUNEL-positive cells was significantly increased in ZDF rats compared with lean animals (Fig. 2D).

Photoreceptors and Cone Bipolar Cells. To assess rod outer segments integrity and photoreceptors and cone bipolar cells morphology, immunostaining for rhodopsin (Rho), an opsin protein in rod outer segments (OSs), and recoverin (Rec), a 23-kDa neuronal calcium-binding protein that is primarily detected in cone and rod photoreceptors and cone bipolar cells, were evaluated. In lean and ZDF rats, Rho immunoreactivity was present in the OS of rods, and Rec was detected in photoreceptors of the ONL and cone bipolar cells in the INL. There were no detectable differences in cellular morphology between animal groups (data not shown).

Glial Cells. To address the degree of glial cell activation, ocular samples were immunostained with antibodies against vimentin (Vim), a type III intermediate filament protein that is
expressed in glial cells, and GFAP, an intermediate filament protein present in glial cells. In control sections, no immunoreactivity was found in any case (Figs. 3A, 3D, 3G). In lean rats, Vim immunoreactivity was detectable throughout the cytoplasm of the Müller cells, from the ILM to the OPL (Fig. 3B), whereas GFAP was limited to the innermost layers of the neuroretina (E). In ZDF rats, Vim was extended to the OLM, with thickened branches at the OPL (C, arrowheads, n = 6). Massive GFAP upregulation was present (F n = 15). Vim and GFAP often colocalize in Müller cells cytoplasm (yellow, H, I). Blue, Hoechst. Scale bar denotes 25 μm.

Table 3. Quantification of Glial Activation Based on Extent of GFAP Staining

<table>
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<th>Score</th>
<th>% Positive GFAP Labeling</th>
</tr>
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<tr>
<td>1</td>
<td>100</td>
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<tr>
<td>2</td>
<td></td>
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<td>3</td>
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<td>4</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
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Glial activation was evaluated by a five-point scoring system based on extent of GFAP staining. Scoring system: Müller cell endfeet region/GCL only (1); Müller cell endfeet region/GCL plus a few proximal processes (2); Müller cell endfeet plus many processes, but not extending to ONL (3); Müller cell endfeet plus processes throughout with some in the ONL (4); Müller cell endfeet plus lots of processes from GCL to outer margin of ONL (5).
close to the OPL and in the GCL/NFL (Figs. 4E, 4F, arrowheads). AGEs immunoreactivity was significantly increased in comparison with lean rats (Fig. 5).

**Choroidal Vessels.** To evaluate choroidal vasculature, ocular sections were immunostained with the *Griffonia simplicifolia* isolecin B4 (IB4), a lectin that specifically binds terminal β-galactosyl residues expressed by various cells including endothelial cells. In control sections, no immunoreactivity was found in any case (Fig. 4G). In both animal groups, IB4 was observed in endothelial cells of the choriocapillaris (Figs. 4H, 4I, arrowheads) and in the small arteries and arterioles of the choroid (H, I, arrows). No differences in vessel morphology were apparent between lean and ZDF rats.

**Retinal Pigment Epithelium.** To study the RPE morphology and cell membrane, RPE cells were examined with antibodies against RPE65, a cytoplasmic protein involved in the production of 11-cis retinal and in visual pigment regeneration, and ZO1, a tight junction protein localized to the RPE membrane. In control sections, no immunoreactivity was found in any case (Figs. 6A, 6D). In both animal groups, RPE65 was observed in endothelial cells of the choriocapillaris, a highly anastomosed network of capillaries forming a thin sheet (Figs. 4H, 4I, arrowheads), and in the vascular region of the choroid, formed by medium and small arteries and arterioles (Figs. 4H, 4I, arrows). No differences in vessel morphology were apparent between lean and ZDF rats.

**DISCUSSION**

With a rapid increase in the prevalence of diabetes mellitus, ocular complications have become a leading cause of blindness in the world since the late 90s. DR is one of the most common ocular complications of diabetes. The vascular features of long-term DR are well documented. Much less
is known of pathologic changes in retinal neurons and glial cells that are likely to occur in DR, given that the disease involves a progressive loss of visual function. In recent years, animal models served to elucidate some of the cellular and molecular mechanisms involved in the ocular pathogenesis of DR and for testing novel treatments. Although many of the models are well characterized in terms of retinal-choroid vascular modifications, morphologic changes in the retina associated with hyperglycemia have not been studied in detail. In the present study, we demonstrated that the spontaneous development of diabetes in ZDF rats results in neuroglia morphologic degenerative changes at 24 weeks of age. Rats are frequently used in in vivo ophthalmology studies owing to a relatively larger tissue size (compared with mice), with which functional assessment and morphologic and molecular analyses can be done. The ZDF rat (fa/fa) genotype displays glucose intolerance and hyperinsulinemia. Retinopathologic studies in these rats mainly focused on the vasculature. Thickening of the capillary basement membrane and modestly increased capillary cell nuclear density were reported. Apoptosis of endothelial cells and pericytes was higher in these rats compared with the lean controls, together with an increased number of acellular capillaries and pericyte ghosts. Complementary electrotoretinography studies of ZDF rats described an unexpectedly higher response of photoreceptors to light stimuli compared with controls, whereas the inner retina developed functional impairment.

In the present study, the ZDF rats rapidly progressed from normoglycemia to hyperglycemia and were clearly diabetic from week 9 to 24. By the end of the experiment, evidence of increased glycation end products was also observed in the ZDF rats because the level of HbA1c was more than double that in the lean controls. The changes in body weight and glycemia in the ZDF rats were consistent with previous studies. At 24 weeks of age, ZDF rats retinas presented with enclosed retinal regions with morphologic degenerative changes. Neuroretina total thickness was significantly increased compared with lean animals, whereas the number of nuclei per row in the nuclear layers was significantly decreased. The cystic changes observed and the increase in neuroretinal thickness could be due to edema from leaky vessels. Johnson et al. did not report abnormal changes in retinal thickness at 16, 23, and 31 weeks old nor in the number of nuclei per row at the ONL and INL at 23 weeks. However, no detailed measurement data were described, and the number of samples evaluated per group was limited (n = 2 to 4 rats per group). Retinal thickening is a common finding in diabetic patients, and transient thickening of the retina could happen before late onset atrophy, as observed in streptozotocin-induced rats after 7.5 months of diabetes. Streptozotocin injection induced type 1 diabetes in different rat strains that have been used as a DR model in several drug testing studies. Ectopic nuclei found in ZDF rats were not characterized in this study; future studies are needed to confirm whether they are ectopic photoreceptors or immune cells. The reduction in the number of cells observed in our study may be linked with photoreceptor cell death via
other apoptosis-dependent or -independent pathways, not evaluated in this study.

Overexpression of GFAP is shown by Müller cells in diabetic patients as well as in the retinas of several animal models of diabetes. In this study, we found significant overexpression of GFAP with increased vimentin expression. Upregulation of GFAP in Müller cells is accepted as an indicator of retinal cell stress. Disturbance of glial cells, cell mediators in the retina water homeostasis, involves the formation of parenchymal edema in diabetes that also leads to retinal thickening. Glial glutamate transporters are essential for keeping the extracellular glutamate concentration below neurotoxic levels. The glutamate-aspartate transporter (GLAST) is the primary glutamate transporter expressed by retinal glial cells. The reduction of GLAST expression in ZDF rats retinas found in this study has also been reported in rats with diabetes induced by streptozotocin; these rats also overexpress other glutamate receptors. In pathologic conditions, the functional downregulation of GLAST induces an increased glutamate concentration in the retina. Increased glutamate can accelerate the death of retinal vascular and nonvascular cells, which might play an important role in the pathogenesis of DR. AGEs are the late products of nonenzymatic glycation only present at moderate levels in glial and vascular structures in nondiabetic human and rat retinas. The levels of these products are much higher in patients with diabetes, and AGEs can also provide the early molecular pathogenic alterations related to disorganization of the junctional complexes of the diabetic retina have been described in patients. Morphologic modifications of choroidal vessels were not observed in ZDF rats at 24 weeks. Other potential retinal vasculature modifications were not evaluated in this study because several authors have previously characterized microvascular changes in ZDF rats. Reliable and appropriate animal models adequately characterized are useful tools for understanding the pathogenesis of human diseases, defining novel therapeutic targets, and screening novel therapeutic drugs. Unfortunately, there is no single animal model that displays all the clinical features of DR as seen in humans from the very early cellular and vascular abnormalities to the proliferative stages. With this study, we contributed to characterizing histologically and immunohistochemically the retinal morphologic modifications in ZDF rats. The pathologic features we characterized in the ZDF diabetic rat retina may present as suitable targets for future therapeutic investigations for the treatment of DR.

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