Retinal Changes in Otsuka Long-Evans Tokushima Fatty Rats (Spontaneously Diabetic Rat)—Possibility of a New Experimental Model for Diabetic Retinopathy

Zhong-Yang Lu*, Imran Ahmed Bhutto and Tsugio Amemiya

*Department of Ophthalmology and Visual Sciences,
Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan*

Purpose: To study retinal changes in the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a spontaneously diabetic rat, and evaluate it as a possible new diabetic retinopathy model.

Methods: We examined the retinas of OLETF rats and Long-Evans Tokushima Otsuka (LETO) rats as controls under both a transmission electron microscope (TEM) and a scanning electron microscope (SEM).

Results: We detected higher blood sugar level in the OLETF rats than in the LETO rats beginning at 5 months of age. The inner nuclear layers of the retina decreased from 3–4 rows to 2 rows, whereas the photoreceptor cell nuclei decreased from 8 rows to 3–6 rows. Retinal pigment epithelial cells decreased in height, and basal infoldings were poorly developed. Retinal capillary basement membranes were significantly thicker in the OLETF rats than in the LETO rats, and endothelial cell damage was observed. The SEM of vascular corrosion casts of OLETF rats showed tortuosity, microaneurysms, and loop formations.

Conclusions: The development of spontaneous hyperglycemia in OLETF rats was associated with alterations in retina ultrastructure. Changes were similar to those seen in diabetic retinopathy, but did not include either hemorrhages or exudates. The OLETF rat may be a useful animal model for the study of ocular complications in humans. **Jpn J Ophthalmol 2003;47:28–35** © 2003 Japanese Ophthalmological Society

Key Words: Corrosion cast, diabetic retinopathy, electron microscopy, OLETF rat, spontaneously diabetic animal model.

Introduction

Diabetic retinopathy is one of the most common causes of vision loss. Despite this, to date, no effective therapy has been developed and the prevention of diabetic retinopathy is not yet possible. One of the reasons for this delay is the absence of a suitable animal model of diabetes mellitus (DM). Diabetes mellitus has been produced experimentally in several animals,[1–5] but these animals rarely develop typical diabetic retinopathy, especially proliferative retinopathy. The diabetic retinopathy seen in animal models consists primarily of vascular changes, which are usually only the initial features of this disease.[6–8] Although these animal models help us to understand the initial vascular pathology of diabetic retinopathy, they are not complete in terms of their ability to reflect the pathology of diabetic retinopathy, nor do they aid in the development of drugs for cure and/or prevention. A model of proliferative diabetic retinopathy is not yet available. Such a model is especially needed for the development of drug therapy, but the first step in attaining this goal is to develop a diabetic retinopathy model. If this first step is

achieved, it will undoubtedly prove useful in the ultimate development of drug therapy for this particular form of retinopathy. The purpose of the present study is to examine the value of the Otsuka Long-Evans Tokushima Fatty (OLETF) rats with spontaneous DM as a new useful model of diabetic retinopathy.

Materials and Methods

Experimental Animals

Four-week-old male OLETF rats (n = 15) were supplied by the Tokushima Research Institute, Otsuka Pharmaceutical Co. Ltd. (Tokushima). The treatment of all animals conformed to the ARVO resolution on the use of animals in research. The rats were housed at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. They were given ad libitum access to water and a conventional diet, F-2 (Funabashi Farms Co., Funabashi).

The rats were weighed and their blood sugar levels were measured monthly using Advantage (Yamanouchi Pharmaceutical Co. Ltd., Tokyo). The urine sugar level was measured monthly using URORABU sticks (Bayer Co Ltd., Tokyo). The Long-Evans Tokushima Otsuka (LETO) (n = 20) rats served as controls.

The eyes of 5 rats of both strains were enucleated each at 14, 17, and 19 months of age under Nembutal anesthesia and the animals were sacrificed.

Transmission Electron Microscope (TEM) Studies of Retina

The enucleated eyes were cut in half at the equator and fixed in 4% glutaraldehyde in 0.05 M cacodylate buffer, and the retina was then cut into six pieces at posterior pole. The specimens were fixed in 4% glutaraldehyde for 1 h, washed in 0.05 M cacodylate buffer overnight, placed in Caulfield osmium fixative for 1 h, dehydrated in a graded series of ethanol baths and embedded in Luveak 812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined under a Hitachi H-300 electron microscope.

Scanning Electron Microscope (SEM) Studies of Retinal Vascular Corrosion Casts

The common carotid arteries of rats of both strains were ligated and plastic cannulas were inserted under Nembutal anesthesia. The jugular veins were then cut. The vascular system was perfused with saline containing heparin sodium (500 IU/100 ml) through the cannulated blood vessels. Immediately after perfusion, Mercox was injected into the cannulated carotid arter-

ies. Then, the eyeballs were enucleated and placed in a warm water bath at 60°C for 4 hours. The ocular tissues were macerated with 20% KOH for 5 days. Microdissection was done to expose the retinal vasculature. The casts were desiccated by freeze-drying and impregnated with osmium, then mounted on SEM stubs and coated with ion spatter gold palladium. The specimens were examined under a Hitachi S-2360N scanning electron microscope at an accelerating voltage of 8 kV.

Measurement of Basement Membrane Thickness of Retinal Capillaries

Five to six capillary sections in the outer plexiform layer were examined in 5 rats of both strains for each animal. Capillaries were photographed at a magnification of $\times 6500$. The basement membrane thickness was measured using a microcaliper. The data were analyzed statistically using the unpaired t-test, and $P < .05$ was taken to indicate significance.

Results

Body Weight

OLETF rats gained weight faster than LETO rats at the beginning of the study, and this difference was significant from 1 to 6 months of age ($P < .001$). However, OLETF rats lost weight after 9 months (Figure 1) and a significant difference in body weight was seen between the two groups after 9 months of age ($P < .001$).
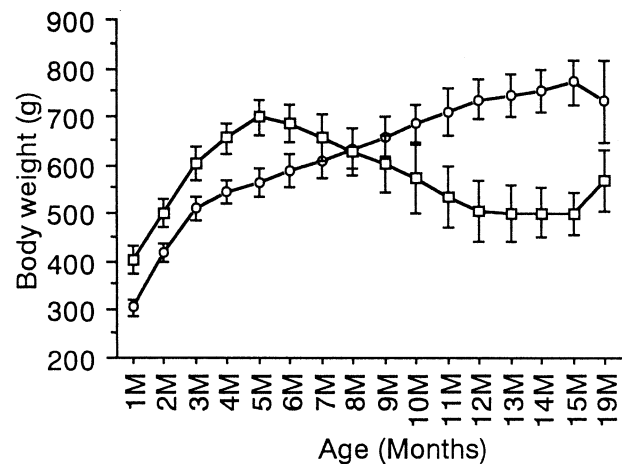
Figure 1. Body weight (mean ± SD). Otsuka Long-Evans Tokushima Fatty (OLETF) rats gained weight faster than Long-Evans Tokushima Otsuka (LETO) rats at the beginning, but lost weight after 9 months. ○: LETO rats, □: OLETF rats.
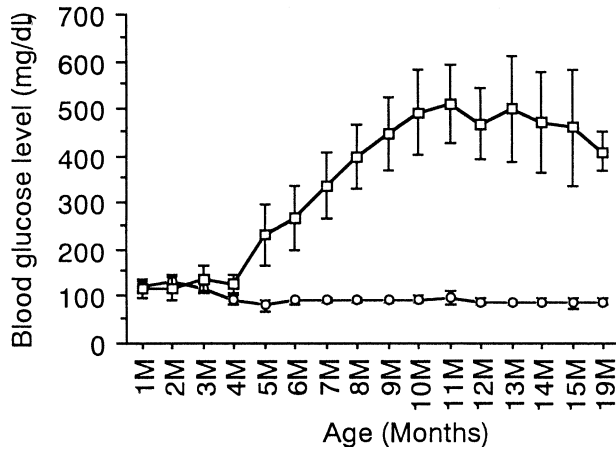
Figure 2. Blood sugar level (mean ± SD). The blood sugar level of Otsuka Long-Evans Tokushima Fatty (OLETF) rats is higher than that of Long-Evans Tokushima Otsuka (LETO) rats starting at 5 months of age, and it is still higher at 19 months. ○: LETO rats, □: OLETF rats.

Blood Sugar Level

The blood sugar level of OLETF rats was higher than that of LETO rats beginning at 5 months of age, and this value increased with age (Figure 2).

Urine Sugar Levels

The urine of OLETF rats showed a positive reaction for sugar starting at 6 months of age.

Cataract

By slit-lamp microscopy, cataracts were detected in almost all of the 19-month-old OLETF rats, whereas no cataracts were found in LETO rats.

TEM Findings

The retinal capillaries of 14-month-old OLETF rats had thickened basement membranes, and their endothelial cell cytoplasm contained many micropinocytic vesicles. The retinal capillaries had pericytes with vacuoles, but no microaneurysms. The photoreceptor cells and retinal pigment epithelium (RPE) appeared to be intact. In 19-month-old OLETF rats, the inner nuclear layer was decreased from 3–4 rows to 2 rows (Figure 3). The photoreceptor cell nuclei were decreased from 8 rows to 3–6 rows (Figure 4) (Table 2). The RPE cells were shortened in height, and the basal infoldings were poorly developed (Figure 5) (Table 3). In the 19-month-old OLETF rats, the retinal capillaries had slightly narrow and irregular lumens. The retinal capil-
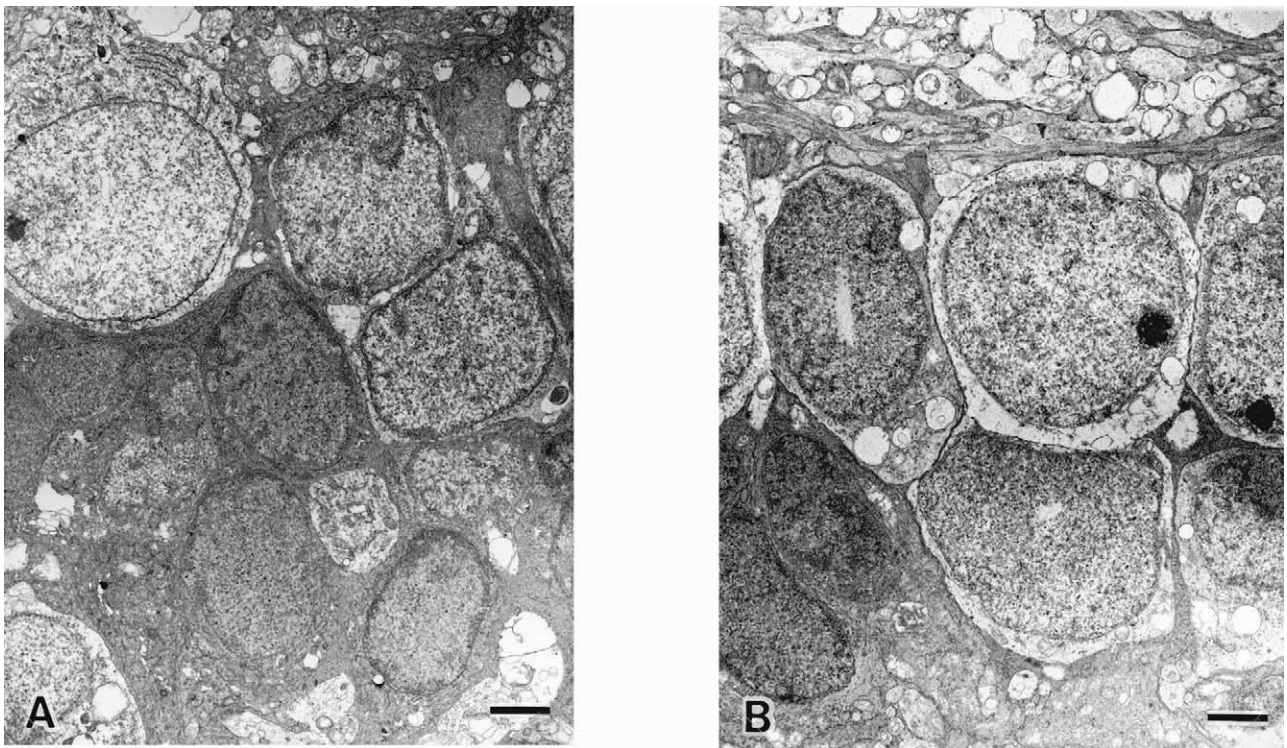
Figure 3. (**A**) Electron micrograph of the inner nuclear layer of a 19-month-old Long-Evans Tokushima Otsuka (LETO) rat. The inner nuclear layer has 3–4 rows. (**B**) The inner nuclear layer of a 19-month-old Otsuka Long-Evans Tokushima Fatty (OLETF) rat. The inner nuclear layer has decreased to two rows. Bar = 2 μm.
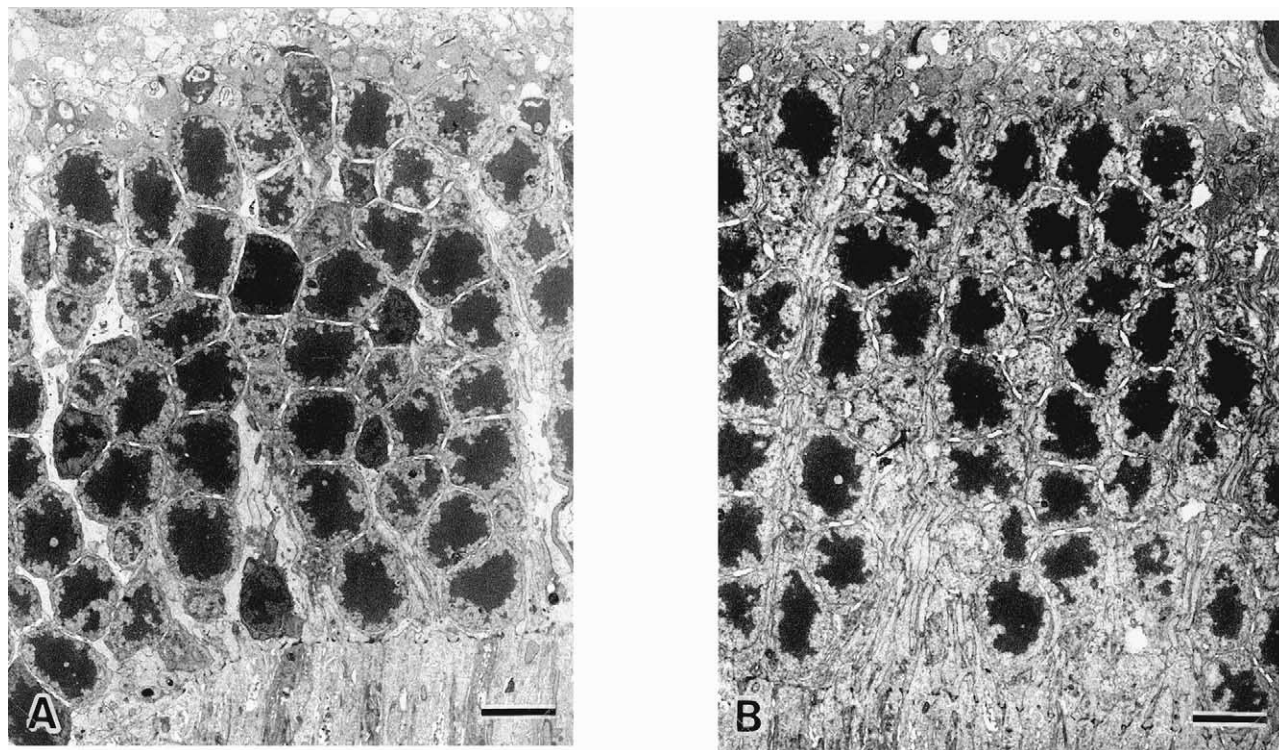
Figure 4. (**A**) Electron micrograph of the photoreceptor cell nuclei of a 19-month-old Long-Evans Tokushima Otsuka rat. The number of photoreceptor cell nuclei is eight rows. (**B**) The photoreceptor cell nuclei of a 19-month-old Otsuka Long-Evans Tokushima Fatty rat. The number of photoreceptor cell nuclei has decreased to 3–6 rows. Bar = 2 μm.

lary basement membranes were significantly thicker in OLETF rats than in LETO rats (Table 1). The capillary lumen was narrow (Figure 6B). Capillary endothelial cells contained vacuoles and some of the cells were thin (Figure 6B). The pericytes of capillaries were swollen and contained vacuoles (Figure 6B). However, hemorrhages, emboli, and exudate were not found.

SEM of Retinal Vascular Corrosion Casts

SEM examination of the retinal vascular corrosion casts of 17- and 19-month-old OLETF rats showed tortuosity and loop formations of the capillaries. Two layers were maintained in the capillary network ar-

chitecture (Figure 7). The retinal vessels were tortuous and narrow, but caliber irregularity was not prominent. Aneurysm and loop formations were found in the retinal capillaries (Figures 7 and 8).

SEM showed no abnormal retinal findings in the control LETO rats (Figure 9).

Discussion

The OLETF rat is a newly developed strain of spontaneously diabetic rats.[9] The characteristic features of OLETF rats[4] are (1) late onset of hyperglycemia; (2) chronic course of the disease; (3) mild obesity; (4) male inheritance; (5) hyperplastic foci of pancreatic islets; and (6) nodular lesions in the kidneys. These characteristic pathological features closely resemble those of human non-insulin-dependent diabetes mellitus (NIDDM).[9]

A good animal model with typical diabetic retinopathy has been sought. Animals with galactose-induced diabetes[1–3] and streptozotocin-induced diabetes[2,4] have been studied for diabetic retinal complications. However, the onset of diabetes in these models is rapid and their life span is generally very short. Even if these rats survive for longer periods of time, proliferative

Table 1. Retinal Capillary Basement Membrane Thickness in the Outer Plexiform Layer

Rat	Mean ± SD (nm)
LETO (19 months)	105.3 ± 29.9 (n = 30)
OLETF (19 months)	290.3 ± 72.9 (n = 30)

P < .001 (Student *t*-test, significant difference). LETO: Long-Evans Tokushima, OLETF: Otsuka Long-Evans Tokushima Fatty, n: examined number of capillaries.
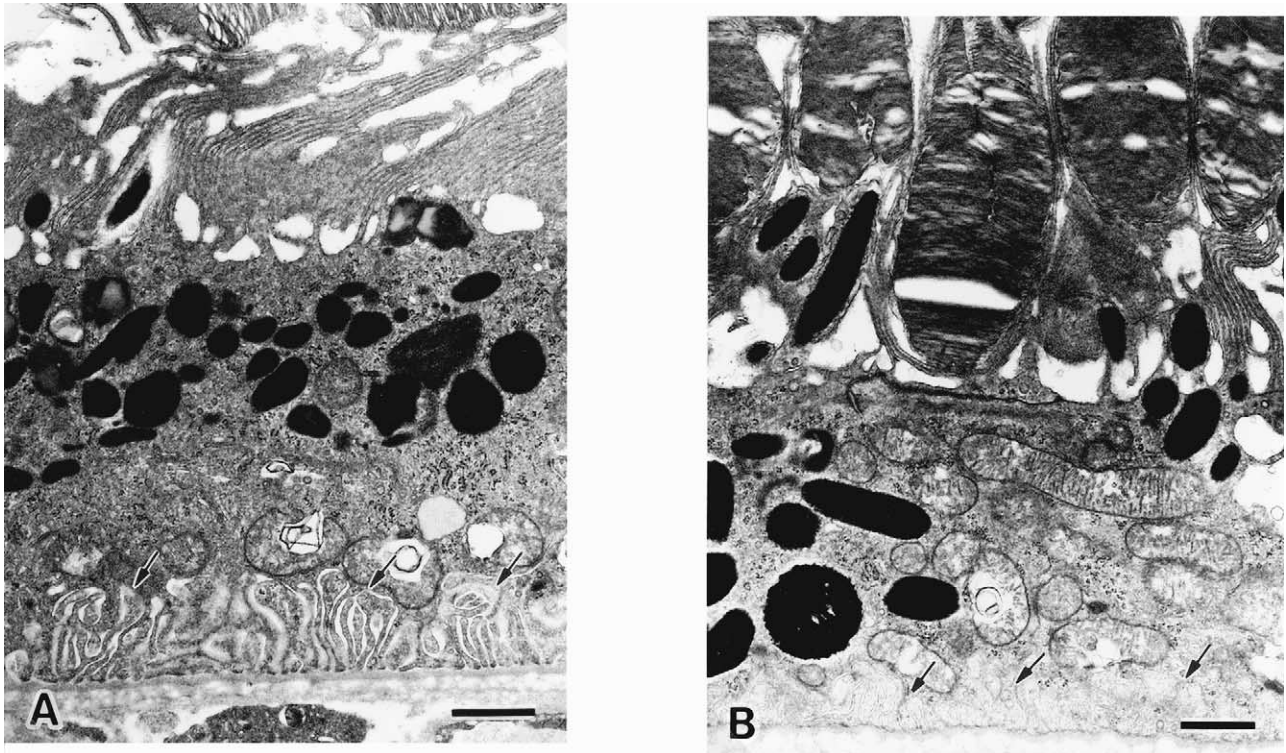
Figure 5. (**A**) Electron micrograph of the retinal pigment epithelium (RPE) of a 19-month-old Long-Evans Tokushima Otsuka rat. (**B**) The RPE of a 19-month-old Otsuka Long-Evans Tokushima Fatty rat. The RPE cells decrease in height and the basal infoldings (arrows) are poorly developed. Bar = 1 μm.

retinopathy is rarely induced. Some researchers[6–8] have used animals with hereditary DM to study diabetic retinal changes, but retinal changes are slight and attention has been focused on the capillaries.

We previously reported retinal capillary changes in OLETF rats under 14 months of age.[10] We were able to prolong the survival of these rats up to 19 months of age, and this allowed for an examination of the details of the retina, and not only the retinal capillaries. Thus it is expected to be a good strain for the study of chronic diabetic complications. In the present study, we detected higher blood sugar level in the OLETF rats than in the LETO rats beginning at 5 months of

age and higher urine sugar level from 6 months of age. OLETF rats gained weight faster than LETO rats from 1 to 7 months. However, OLETF rats lost weight after 9 months of age, and all OLETF rats also developed cataracts.

Various retinal changes have been consistently reported in humans with diabetes[11,12] and some diabetic animal models.[1,5,13–15] OLETF rats showed similar capillary changes. Fourteen-month-old OLETF rats showed tortuosity and loop formation of capillaries, but no microaneurysms or changes in photoreceptor cells or RPE.[9] These latter two changes first appeared at the age of 17 months. TEM revealed significant dif-

Table 2. Number of Nuclei in Outer and Inner Nuclear Layers (Mean ± SD)

Rat	Inner Nuclear Layer Nuclei in 30 × 30 μm	Outer Nuclear Layer Nuclei in 20 × 20 μm
LETO (19 months)	11.3 ± 0.34* (n = 30)	45.1 ± 0.31* (n = 30)
OLETF (19 months)	6.7 ± 0.26* (n = 30)	35.1 ± 0.48* (n = 30)

LETO: Long-Evans Tokushima, OLETF: Otsuka Long-Evans Tokushima Fatty, n: number of specimens examined.

*$P < .0001$ (Student t-test, significant difference).

Table 3. Height of Retinal Pigment Epithelium (RPE) Cells and Basal Infoldings (Mean ± SD, μm)

Rat	RPE	Basal Infoldings
LETO (19 months)	5.233 ± 0.035* (n = 30)	1.200 ± 0.036* (n = 30)
OLETF (19 months)	4.274 ± 0.025* (n = 30)	0.583 ± 0.050* (n = 30)

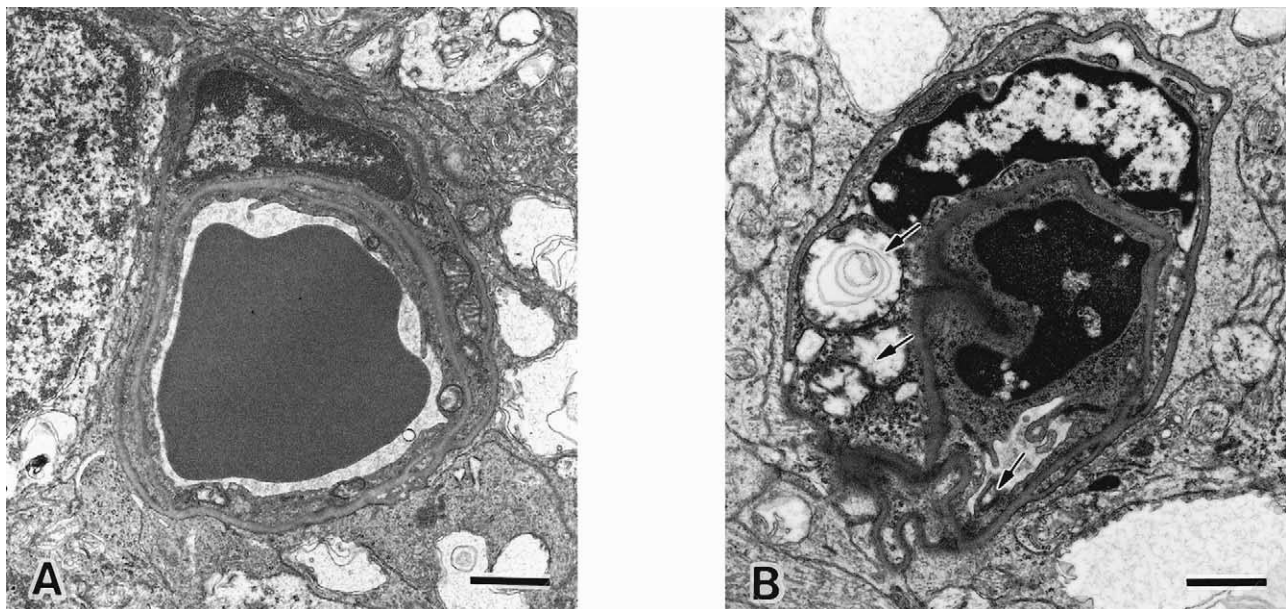LETO: Long-Evans Tokushima, OLETF: Otsuka Long-Evans Tokushima Fatty, n: number of specimens examined.

*$P < .001$ (Student t-test, significant difference).

ferences in basement membrane thickness and capillary endothelial cell degeneration between 14-month-old OLETF rats and age-matched control LETO rats. These capillary changes occur in the early stage of human diabetic retinopathy. SEM of vascular corrosion casts demonstrated tortuosity, microaneurysms, and loop formations in retinal capillaries at the age of 17 months. TEM also detected a decrease in the inner nuclear layer from 3–4 rows to 2 rows; a decrease in the photoreceptor cell nuclei in the outer nuclear layer from 8 rows to 3–6 rows; a decrease in the height of the RPE cells and a decrease in the amount of basal infoldings.

The trypsin digestion method is very useful for retinal vascular studies in that it allows for demonstration of pericyte changes and acellular blood vessels. However, the present authors preferred retinal vascular casts and the SEM method to demonstrate

the architecture, branching, density, caliber irregularity, crossing phenomena, and caliber with a three-dimensional view. However, the cast and SEM method does not show acellularity or endothelial changes. To overcome this deficit of casts with the SEM method, TEM was used to demonstrate the capillary structure and showed the findings corresponding to acellular blood vessels and pericyte changes.[15]

The corrosion cast technique has some potential problems, including incomplete corrosion, incomplete filling, and extravasation of injected resin. Occasionally, there was a leakage of plastic material or incomplete filling of retinal capillaries, perhaps due to differences in physiological pressure between vessels and capillaries.[16] In the present study, a microaneurysm-like feature was taken. It was uncertain whether this feature was due to a leakage of resin. However, the authors believe it to be a microaneurysm because
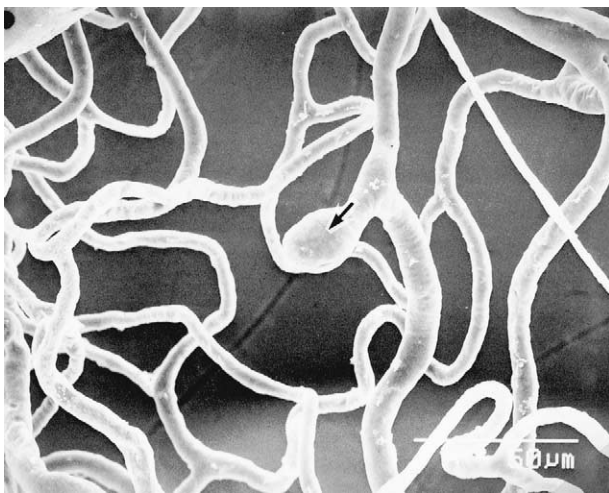

Figure 6. (**A**) Electron micrograph of the retinal capillary of a 19-month-old Long-Evans Tokushima Otsuka rat. (**B**) The retinal capillary of a 19-month-old Otsuka Long-Evans Tokushima Fatty rat. The capillary lumen is narrow. The pericyte and endothelial cells have vacuolated mitochondria and vacuoles (arrows) in the cytoplasm. Bar = 1 μm.
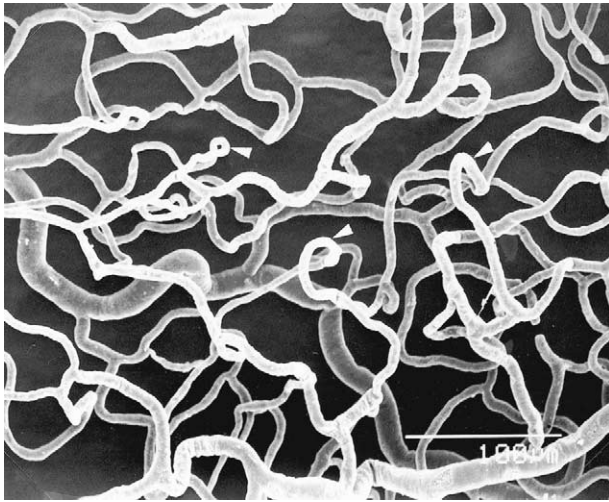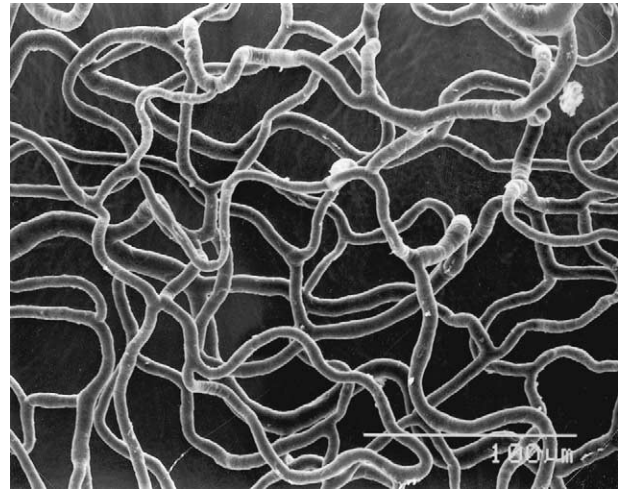
Figure 7. Scanning electron micrograph of retinal vascular corrosion cast of a 17-month-old Otsuka Long-Evans Tokushima Fatty rat. The capillaries show tortuosity and loop formations (arrow heads). Magnification: ×200.

Figure 9. Scanning electron micrograph of the retinal vascular corrosion cast of a 17-month-old Long-Evans Tokushima Otsuka rat. The capillaries are regularly and densely arranged in two layers and have remarkably uniform caliber. Magnification: ×200.

we rarely see such figures in casts, whereas casts of OLETF occasionally showed a few such figures in the whole view of the retinal vasculature. If many such figures were seen, this can reasonably be concluded to be an artifact.

The photoreceptor cell findings are not clearly described in the current textbooks, probably because the main causative factors are vascular. However, nonvascular retinal changes are described in the old literature. In effect, the diabetic retina shows a thinned

Figure 8. Scanning electron micrograph of retinal vascular corrosion cast of a 17-month-old Otsuka Long-Evans Tokushima Fatty rat. A microaneurysm (arrow) is present. Magnification: ×600.

nerve fiber layer and a decrease in the number of ganglion cells; the light microscopic photographs of diabetic retinas show a decrease in the outer and inner nuclear layers.[17] These findings in human diabetic retinopathy are similar to those seen in OLETF rat retinas, which have not been described previously in animal models of diabetes mellitus. This is probably because most of the researchers have paid attention only to the retinal vasculature, and follow-up studies have been too short to see the changes of diabetic retinopathy that develop slowly. Therefore, OLETF rats are valuable in the study of diabetic retinopathy. Barber et al reported an increase of neural cell apoptosis in the retinas of diabetic patients and of streptozotocin-induced diabetic rats and indicates that neurodegeneration is an important component of diabetic retinopathy.[18] In the present study, no electrophysiological studies were carried out, and thus we were unable to assess function. However, the formation of severe cataracts and severe pathological changes in the retina allow us to assume impaired vision in this animal. The electroretinogram (ERG) and dark adaptation in diabetic patients demonstrate abnormal reactions.[19, 20] These abnormalities suggest that the functions of the photoreceptor cells, bipolar cells, and amacrine cells are disturbed. Yonemura et al[19] reported that abnormal oscillatory potentials appeared prior to morphological changes in human diabetic retinopathy. According to Shirao et al,[21] OLETF rats first showed delayed oscillatory potentials at 50 weeks of age, but the present study failed to show any ultrastructural

changes before 14 months of age. The later appearance in the retina of diabetic morphological changes than the ERG changes is not inconsistent with the findings in previous reports. Thus, the retinal changes of OLETF rats demonstrated by both histological and electrophysiological studies may not be incompatible with simple diabetic retinopathy, especially in its initial stage.[19,21] Although diabetic retinopathy is an angiopathy, neuroretinal changes should receive more attention in diabetic retinopathy. Differences from characteristics of human diabetic retinopathy are the absence of hemorrhages and exudates. In this respect, OLETF rats are not satisfactory for the study of diabetic retinopathy. It seems to be difficult for rats to have retinal hemorrhages or exudates. For instance, the spontaneously hypertensive rat never shows hemorrhages or exudates in the retina, although retinal capillaries are typical for hypertension. Considering the natural course of the disease in this rat, however, it is most likely that the diabetic retinopathy in this spontaneously diabetic rat is similar to a diabetic retinopathy.

The present paper is the first detailed description of the morphological changes in the retinal capillaries, the RPE, and the inner and outer nuclear layers of OLETF rats. We hope that the findings in this study will be helpful in determining the suitability of the OLETF rat as an animal model for further diabetic retinopathy studies that will lead to the development of effective therapy for this disease.

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