Death of Retinal Neurons in Streptozotocin-Induced Diabetic Mice

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PURPOSE. Neuronal cell death has been reported in retinas of humans with diabetic retinopathy and in diabetic rat models. Little is known about neuronal cell death in mouse models of diabetic retinopathy. This study was designed to determine whether neurons are lost in diabetic mouse retinas and whether the loss involves an apoptotic process.

METHODS. Three-week-old C57Bl/6 mice were made diabetic with streptozotocin. They were studied over the course of 14 weeks after onset of diabetes. Eyes were processed for morphometric analysis and detection of apoptotic cells by TUNEL analysis and activated caspase-3 and were subjected to electron microscopy.

RESULTS. Morphometric analysis of retinal cross sections of mice that had been diabetic 14 weeks showed ~20% to 25% fewer cells in the ganglion cell layer compared withagematched control mice. There was a modest, but significant, decrease in the thickness of the whole retina and the inner and outer nuclear layers in mice that had been diabetic for 10 weeks. TUNEL analysis and detection of active caspase-3 revealed that cells of the ganglion cell layer were dying by apoptosis. Electron microscopic analysis detected morphologic features characteristic of apoptosis, including margination of chromatin and crenated nuclei of cells in the ganglion cell layer.

CONCLUSIONS. The data suggest that in diabetic mouse retinas, neurons in the ganglion cell layer die, and this death occurs through an apoptotic pathway. Diabetic mice may be appropriate and valuable models for studies of neuronal cell death in diabetes. (Invest Ophthalmol Vis Sci. 2004;45:3330–3336) DOI:10.1167/iovs.04-0247

The present study examined neuronal cell loss in retinas of diabetic mice. It has become increasingly clear that diabetic retinopathy affects not only retinal vasculature, but also retinal neuronal and glial cells.1–4 Electrophysiologic studies of human patients with diabetes suggest alterations in the neural retina, such as loss of color and contrast sensitivity, within 2 years of diabetes onset.5,6 Focal ERG analyses, which can detect electrical responses of ganglion cells, reveal dysfunction of these cells early in diabetes.7,8 Analyses of retinal tissue samples from diabetic patients provide further support for the involvement of retinal neurons in diabetic retinopathy. Two studies in the 1960s described loss of retinal neurons in diabetic patients.9,10 Recently, Barber et al.11 analyzed the retinas of diabetic patients by the TUNEL method and observed significantly more apoptotic neurons in retinas of the patients than in control subjects. Similar results were observed by Bek.12 Kerrigan et al.,13 focusing on ganglion cell death in primary open-angle glaucoma, also analyzed eyes of subjects with diabetes (but no glaucoma) and found more TUNEL-positive ganglion cells in these tissues than in retinas of nondiabetic control subjects.

Rodent models have been used to elucidate the mechanisms of retinal cell damage in diabetes. Rats have been used extensively for analysis of vascular and nonvascular alterations. Several groups have established that there are significantly more neuronal cells undergoing apoptosis, particularly in the ganglion cell layer (GCL), in retinas of diabetic rats than in control animals.11,14–17 Others have observed loss of the axonal fibers in diabetic rat retinas.18–21 ERG studies performed in diabetic rats have detected reduced ERG responses as early as 2 weeks after onset of diabetes.22,23 Mohr et al.24 reported increased levels of caspase activation, a marker of apoptosis, in retinas of diabetic mice. In a recent report comparing neuroretinal changes in the rat and mouse, investigators detected ganglion cell death in the rat diabetic model, but not in the mouse model.17 That is, when whole-mounted retinas were analyzed for incidence of apoptosis using the TUNEL method, there appeared to be no difference in the number of apoptotic retinal neurons in diabetic mice compared with nondiabetic control mice. To our knowledge, there have been no other studies reported about neuronal cell loss in retinas of diabetic mice. We found this possible species difference in neuronal death between diabetic mice and rats intriguing, especially in light of the report by Kowluru25 in which metabolic changes in retinas of diabetic mice were very similar to those in diabetic rats. Both species showed increased oxidative stress, PKC activity, and nitric oxide (NO) levels in the retina. Given the extraordinary usefulness of mice as models of disease, we sought to explore in detail retinal neuronal cell loss in diabetic mice. In the present study, we systematically analyzed early changes in the mouse retina as a consequence of diabetes, applying several assessments of apoptosis and morphometric analyses. These studies showed that the cells in the retinal GCL of the STZ-induced diabetic mouse undergo apoptosis.

MATERIALS AND METHODS

Reagents

Reagents used in these studies were from the following sources: polyclonal antibody against caspase-3 (R&D Systems, Minneapolis, Minnesota), and 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO). Reagents used in these studies were from the following sources: polyclonal antibody against caspase-3 (R&D Systems, Minneapolis, Minnesota), and 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO).
MN); anti-rabbit IgG conjugated to FITC, normal goat serum (Jackson Immunoresearch, West Grove, PA); goat anti-rabbit IgG conjugated to Cy5 (Molecular Probes, Eugene, OR); antifade mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA); optimal cutting temperature (OCT) compound (Tissue Tek; Miles Laboratories, Elkhart, IN); in situ apoptosis detection kit with fluorosecin (ApopTag; Intergen, Purchase, NY); STZ (N-[methylnitroso-carbamoyl]-D-glucosamine) and all other agents (Sigma-Aldrich, St. Louis, MO). The urine strip test was from American Diagnostics (Minneapolis, MN), and the glucometer (Prestige Smart System) was from Home Diagnostics (Woonsocket, RI).

Induction of Experimental Diabetes in Mice

C57BL/6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and were maintained in our colony, as described previously.26 Diabetes was induced chemically in 3-week-old C57BL/6 mice, according to the method of Phelan et al.27 Mice received an intraperitoneal injection of 75 mg/kg STZ dissolved in sodium citrate buffer (0.01 M, pH 4.5) on three successive days. Mice were screened for diabetes approximately 20 to 300 µm apart. These measurements were then averaged to yield a measurement for that particular section. For each animal examined, three separate eye sections were measured. All measurements were obtained with a microscope and digital camera (Axioskop; Carl Zeiss, Inc., Oberkochen, Germany; camera equipped with Spot Software ver. 4.0.2; Diagnostic Imaging, Sterling Heights, MI).

Morphometric Evaluation and Measurement Procedures

Morphometric evaluation of retinas included scanning tissue sections for evidence of gross disease followed by morphometric analysis, which included measurements of the thickness of the total retina, the thickness of the outer nuclear layer, the thickness of the inner nuclear layer, and the number of cells in the GCL. The number of cells in the GCL was quantified by counting cells from the temporal to the nasal ora serrata. Thickness measurements were made in the posterior retinas at four points, two on either side of the optic nerve that were approximately 200 to 300 µm apart. These measurements were then averaged to yield a measurement for that particular section. For each animal analyzed, three separate eye sections were measured. All measurements were obtained with a microscope and digital camera (Axioskop; Carl Zeiss, Inc., Oberkochen, Germany; camera equipped with Spot Software ver. 4.0.2; Diagnostic Imaging, Sterling Heights, MI).

In Situ Detection of DNA Fragmentation by TUNEL Assay

The TUNEL assay was performed using the in situ apoptosis detection kit with fluorescein, according to our published method.26 Tissues were viewed by epifluorescence by using standard fluorescence excitation and emission filters. Each section was scanned systematically from the temporal to the nasal ora serrata for fluorescent cells indicative of apoptosis. To distinguish between structures that autofluoresced versus those that were TUNEL positive, all slides were examined first with the rhodamine filter and then with the FITC filter. Autofluorescent structures were visible under both filters, whereas TUNEL-positive cells were detectable only with the FITC filter. Positively labeled cells were counted in the GCL.

Immunohistochemical Detection of Active Caspase-3

Immunohistochemical methods were performed on cryosections for the detection of active caspase-3. Cryosections of eyes were fixed in ice-cold acetone for 5 minutes, washed with 0.01 M PBS (pH 7.4) and blocked with 4% normal goat serum for 90 minutes. They were incubated with the primary polyclonal antibody against active caspase-3 (1:250) overnight at 4°C. Negative control sections were treated identically with buffer only or normal rabbit serum. Sections were rinsed and incubated for 1 hour with anti-cy3 antibody (1:500). Tissues were viewed by epifluorescence using standard fluorescence excitation and emission filters. Each section was scanned systematically from the temporal to the nasal ora serrata for fluorescent cells indicative of cells undergoing apoptosis. As in the TUNEL assay, the positive cells were counted.

Image Capture and Data Analysis

Images from the TUNEL assay and immunohistochernical studies of active caspase-3 were obtained with a fluorescence microscope (Axioskop 2; Zeiss) equipped with a digital camera and software (Spot camera and software ver. 4.0.2; Diagnostic Imaging). Analysis of variance was used to determine whether there were significant differences in morphologic measurements and in the number of TUNEL- and caspase-3-positive cells in diabetic versus nondiabetic, age-matched control mice. P < 0.05 was considered significant. Tukey’s paired comparison test was the post hoc statistical test. Measurements obtained in the morphometric analysis of these retinas were analyzed by ANOVA (significance level: P < 0.05, The Tukey post hoc test).

RESULTS

Three-week-old C57BL/6 mice were made diabetic by injection of 75 mg/kg STZ. Table 1 shows the body weight and blood glucose levels for diabetic and age-matched control mice. Diabetic mice had a 36% gain in weight from 2 to 14 weeks after onset of diabetes, whereas age-matched controls had a 42% gain in weight. By 14 weeks after onset of diabetes, diabetic mice weighed significantly less than control mice. Blood glucose levels differed significantly between diabetic and control mice at all ages studied.

Morphometric Evaluation of Diabetic Retinas

Before eyes of diabetic mice were subjected to systematic morphometric analysis, the retinas were examined for evidence of gross disease, such as rosettes within the nuclear layers. There was no evidence of gross retinal disease in diabetic mouse retinas up to 14 weeks after onset of diabetes. Systematic morphometric examination of hematoxylin and eosin–stained retinal cryosections showed no significant differences in the total retinal thickness of diabetic mouse retinas compared with nondiabetic, age-matched control retinas at 2, 4, 6, and 8 weeks after onset of diabetes (Fig. 1A). By 10 to 12
weeks, however, the thickness of retinas of diabetic mice was significantly less than that in age-matched control mice (Fig. 1A). Specifically, the thickness of the outer and inner nuclear layers was reduced significantly in diabetic mice at 10 to 12 weeks after onset of diabetes (Figs. 1B, 1C). There did not appear to be a significant difference in the thickness of the inner or outer plexiform layers between diabetic and control mice (data not shown).

Additional measurements were made of the number of cells in the GCL of diabetic and control mice. A hematoxylin and eosin–stained section of the retina of a control mouse age matched to a mouse that had been diabetic for 12 weeks is shown in Figure 2. Retinas of control mice had a GCL in which the cells were densely packed. There was typically a uniform distribution of cells. Except for occasional blood vessels, there was little space intervening between cells. In contrast, diabetic retinas demonstrated a loss of cells in the GCL. There were areas where the distribution of ganglion cells appeared normal adjacent to areas devoid of any cells. The arrow in Figure 2B indicates a region of cellular dropout.

To determine whether there is a significant difference in the number of cells in the GCL of diabetic versus control mice, the cells were counted in sections of eyes from the temporal to the nasal ora serrata. As shown in Figure 3, control retinas typically had ~500 to 550 cells in cross sections of this layer throughout all ages studied. During the first few weeks of diabetes, the number of cells in the GCL did not differ significantly from control animals. However, by 10 to 14 weeks after onset of diabetes, there were significantly fewer (~20%–25%) cells in the GCL in diabetic retinas compared with control retinas ($P < 0.05$). When data were expressed as number of cells per 100-μm length of retina, there were significantly fewer cells in 10-week diabetic mice (10.31 ± 0.5 cells/100 μm retinal length) than in age-matched, nondiabetic mice (15.8 ± 0.6 cells/100 μm retinal length).

**TUNEL Analysis and Detection of Active Caspase-3**

To characterize the cell loss observed in the GCL of diabetic mice, we applied two assays of cell death, the TUNEL assay and detection of active caspase-3. TUNEL analysis detects cells in which DNA is fragmenting and, though used widely as a marker for apoptosis, also reflects cells dying by necrosis.\(^2\)\(^6\) Active caspase-3 is a marker of apoptosis.\(^2\)\(^7\) Examination of retinas of diabetic mice showed many TUNEL- and caspase-3–positive cells in the GCL, whereas control mice had few positive cells. Photomicrographs of retinas processed for the TUNEL assay and detection of active caspase-3 are shown in

![Figure 1](http://example.com/fig1.png) **Figure 1.** Morphometric analysis of diabetic and age-matched, nondiabetic control mouse retina. Hematoxylin and eosin-stained cryosections of retinas from diabetic and age-matched control retinas were subjected to morphometric analysis at 2, 4, 6, 8, 10, and 12 weeks after onset of diabetes. (A) Total retinal thickness. (B) Thickness of the outer nuclear layer (ONL). (C) Thickness of the inner nuclear layer (INL). Data are the means ± SE of measurements in retinas of four control and six diabetic mice. *Significantly different from control ($P < 0.05$).

![Figure 2](http://example.com/fig2.png) **Figure 2.** Light microscopic evaluation of diabetic and age-matched, nondiabetic control retina. Hematoxylin and eosin–stained cryosections of retinas of control (A) and diabetic (B) mice (12 weeks after onset of diabetes). The cells of the GCL are uniformly distributed in the control mouse, whereas there are areas of cellular dropout (arrow) in the diabetic retina. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigment epithelium.
Figure 4. Cryosections of eyes from mice killed 2, 6, and 12 weeks after onset of diabetes and age-matched control sections were analyzed to determine the number of TUNEL and active caspase-3–positive cells. In the results of both assays, there were significantly more positive cells in the GCL of retinas of diabetic mice than in control mice. Data for the analysis of caspase-3 are shown in Figure 5. Of note, the presence of TUNEL-positive and active caspase-3–positive cells was not limited to the GCL. The inner nuclear layer demonstrated occasional positive cells, as did the outer nuclear layer of photoreceptor cell nuclei, at all ages studied (data not shown).

Electron Microscopic Analysis of Diabetic Retinas

Retinal sections were examined systematically to determine whether the neurons of the GCL exhibit ultrastructural features characteristic of apoptotic cell death. Figure 6 shows light micrographs of toluidine blue-stained, Epon-embedded
were unremarkable in control eyes. The toluidine blue staining of the cells was a pale, mottled blue. Blood vessels uniform in size and distribution (Fig. 6A). The toluidine blue onset of diabetes). In control retinas, the cells of the GCL were

![Figure 6. Ultrastructural analysis of diabetic mouse retina. Light photomicrographs of toluidine blue-stained Epon-embedded retinal sections of control (A) and diabetic mice (4 weeks after onset of diabetes; B). Arrows: deeply stained, shrunken cells; arrowhead: a vascular tuft emerging through the GCL into the nerve fiber layer. Electron micrograph of cells in the GCL of diabetic mouse retina that were deeply stained and shrunken (C) adjacent to cells within the same retina that were normal in appearance (D). Magnification: (A, B) ×400; (C, D) ×7700.](image)

retinal sections of control and diabetic eyes (4 weeks after onset of diabetes). In control retinas, the cells of the GCL were uniform in size and distribution (Fig 6A). The toluidine blue staining of the cells was a pale, mottled blue. Blood vessels were unremarkable in control eyes. The toluidine blue-stained sections of a retina of a diabetic mouse 4 weeks after onset of diabetes showed an increased number of darker-staining (deep purple), shrunken cells reminiscent of dying cells (Fig. 6B). The blood vessels were much more noticeable in the diabetic retina and appeared as small tufts emerging through the GCL and projecting into the nerve fiber layer. Dark, shrunken cells in the GCL and small vascular tufts were observed in 12 of 12 eyes of mice that were examined at 4 weeks after onset of diabetes. Ultrastructural analysis of the shrunken cells in the GCL revealed the classic morphologic characteristics of apoptosis (including margination of chromatin and crenated nuclei; Fig. 6C). As is typical of apoptosis, adjacent cells within the same layer appeared normal (Fig. 6D). Cells of the GCL in control mice had uniformly normal-appearing nuclei when examined at the ultrastructural level (data not shown). Ultrastructural examination of other cell types in the diabetic retinas revealed occasional evidence of apoptosis, particularly in the inner nuclear layer (data not shown).

**DISCUSSION**

In the present study, we examined neuronal cell death in retinas of diabetic mice. Two important findings emerged from this work. The first is that diabetic mice demonstrated a significant loss of cells in the GCL during diabetes. Retinas of diabetic and age-matched control mice were analyzed morphometrically by measuring the thickness of various retinal layers and by counting the number of neurons in the GCL. Initially, the thickness of the inner and outer nuclear layers did not differ significantly between diabetic and nondiabetic mice. By 10 weeks after onset of diabetes, however, both inner and outer nuclear layers were significantly thinner in retinas of diabetic compared with control mice. In studies of rat models of diabetes, Barber et al. reported significant differences in the thickness of the inner nuclear layer as a consequence of diabetes. Park et al. reported thinning of the inner nuclear layer and marked thinning of the outer nuclear layer by 24 weeks after onset of diabetes. Similar to the study by Barber et al., was our finding that the number of neurons in the GCL decreased significantly in retinas of diabetic mice as the diabeteses progressed compared with age-matched control mice. Our data presenting the number of neurons in retinal cross sections (Fig. 3) provide strong evidence that neurons are lost in the GCL of diabetic mice. Our observation that the inner and outer nuclear layers were thinner in diabetic mice suggests neuronal cell loss in these layers as well.

The second important finding was that neurons of the GCL in diabetic mice were dying by apoptosis. This conclusion is based on several assays for apoptosis. In our study, we used 10-μm-thick frozen sections for the TUNEL assay and detection of active caspase-3. In addition, we embedded retinas in Epon for study by electron microscopy, as apoptosis is classically defined by its morphologic features. In both preparations (frozen and plastic-embedded sections), it was clear that cells of the GCL were affected. TUNEL assays and assays to detect caspase-3, both markers of apoptosis, showed significantly more positive cells in this layer in diabetic mouse retinas than in age-matched control retinas. In addition, electron microscopic studies revealed cells in the GCL that had the classical morphologic characteristics of apoptosis, whereas such features were rarely observed in nondiabetic control retinas. A recent paper, Asnaghi et al. examined TUNEL-positive cells in diabetic rat retinas and found a fourfold increase in the number of apoptotic neurons. They also examined diabetic mouse retinas and concluded that neural apoptosis was not a feature of the diabetic mouse retina. In that report, wholemounted retinas (rather than retinal cross sections) were subjected to TUNEL assay and examined by fluorescence. The TUNEL-positive cell count data were presented, and analysis of four retinas at 10 weeks’ duration of diabetes and five retinas at 24 weeks’ duration revealed no difference in TUNEL-positive cells. Our data (Fig. 4B) from TUNEL analysis of representative sections of mouse retina (6 weeks diabetic) showed several TUNEL-positive cells in the GCL. One significant difference between the two studies was that we did not maintain our mice on insulin, whereas Asnaghi et al. used a low dosage of insulin to prevent weight loss. No data about the number of neurons in the retinas of diabetic mice were provided; thus, we cannot compare this aspect of our study with that of Asnaghi et al.

The data obtained from these studies suggest that mice are a valid model for studies of neuronal cell death in diabetic retinopathy, consistent with findings in other species. The
work is not merely an extension of an observation to an additional species, however, because of the profound usefulness of mice in experimental biology. There are many transgenic and knockout mouse strains available in which specific genes are nonfunctional. Using strains in which the nonfunctional gene encodes a protein thought to play a role in the complications of diabetes, one can induce diabetes and test whether the combined effects of the lack of gene function coupled with diabetes accelerates, delays, worsens, or prevents the retinal diabetic phenotype. Mouse retinas could be examined for vascular changes, described by others, as well as neuronal changes observed in the present study. An effort to characterize retinal phenotypes in various mutant mouse strains, which have been made diabetic, has gotten under way recently through the Animal Models of Diabetic Complications Consortium (http://www.amdcc.org).

In the present work, we did not attempt to determine the cause of neuronal cell death in the diabetic mouse retina. Recent reports of elevation of glutamate in the vitreous of humans with diabetes\textsuperscript{32} and in rat models\textsuperscript{33,34} would justify investigations of the role of this excitatory amino acid in the neuronal cell death observed in diabetic mice. Observations from our laboratory that the intravitreal injection of excitatory amino acid homocysteine leads to death of cells in the GCL\textsuperscript{25} may suggest another possible mechanism of cell death in diabetic retinopathy, given the possible association of elevated levels of homocysteine in diabetes and diabetic retinopathy.\textsuperscript{35,37} The potential role of increased oxidative stress, PKC activity, and nitric oxide (NO) levels in the retina would be other logical areas to investigate as mediators of neuronal cell death in diabetic mouse retinas.\textsuperscript{25,38,39} The abundance of mouse models having mutations in relevant genes will permit elegant studies of the interaction of diabetes with other genes in the development of neuronal cell death in diabetic mice.

References

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