Neural Apoptosis in the Retina during Experimental and Human Diabetes

Early Onset and Effect of Insulin

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Abstract

This study determined whether retinal degeneration during diabetes includes retinal neural cell apoptosis. Image analysis of retinal sections from streptozotocin (STZ) diabetic rats after 7.5 months of STZ diabetes identified 22% and 14% reductions in the thickness of the inner plexiform and inner nuclear layers, respectively (P < 0.001). The number of surviving ganglion cells was also reduced by 10% compared to controls (P < 0.001). In situ end labeling of DNA terminal dUTP nick end labeling (TUNEL) identified a 10-fold increase in the frequency of retinal apoptosis in whole-mounted rat retinas after 1, 3, 6, and 12 months of diabetes (P < 0.001, P < 0.001, P < 0.01, and P < 0.01, respectively). Most TUNEL-positive cells were not associated with blood vessels and did not colocalize with the endothelial cell-specific antigen, von Willebrand factor. Insulin implants significantly reduced the number of TUNEL-positive cells (P < 0.05). The number of TUNEL-positive cells was also increased in retinas from humans with diabetes. These data indicate that retinal neural cell death occurs early in diabetes. This is the first quantitative report of an increase in neural cell apoptosis in the retina during diabetes, and indicates that neurodegeneration is an important component of diabetic retinopathy. (J. Clin Invest. 1998. 102:783–791.) Key words: cell death • diabetic retinopathy • retinal ganglion cells • neurons, neuroglia

Introduction

Diabetic retinopathy is the most common complication of diabetes, affecting over 90% of persons with diabetes and progressing to legal blindness in about 5%. The vascular features of long-term diabetic retinopathy are well documented (1). Much less is known of pathological changes in retinal neurons that are likely to occur in diabetic retinopathy, given that the disease involves a progressive loss of visual function.

The pathologic vascular lesions associated with diabetic retinopathy have been described using the trypsin digest prepara-

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onset of diabetes; and (2) which cells, other than vascular cells, become apoptotic? The following study quantifies apoptosis in the retinas of streptozotocin diabetic rats and in postmortem retinas of humans with diabetes. We report that neural cell apoptosis is increased after 1 mo of diabetes and remains elevated over a 12-mo period. Furthermore, retinal apoptosis is reduced by insulin. There are overall reductions in the thickness of the inner layers of the retina, accompanied by a reduction in the number of ganglion cells in rat retinas after long-term experimental diabetes. These findings reveal that retinal neuron and glial cell degeneration occurs early on in the course of diabetes and should be considered an important component of the pathology of diabetic retinopathy.

Methods

Animals. Male Sprague-Dawley rats weighing 150–175 g were purchased from Charles River Laboratories (Wilmington, MA) and housed in the Penn State University College of Medicine animal facility in accordance with the Institutional Animal Care and Use Committee guidelines, and the Association of Research in Vision and Ophthalmology Resolution on the Care and Use of Laboratory Animals. All rats were group housed in suspended wire bottom cages with ad libitum food and water and under a normal 12-h light/dark schedule. Diabetes was induced by a single tail vein injection of streptozotocin (STZ; Sigma, St. Louis, MO) (65 mg/kg freshly dissolved in citrate buffer, pH 4.5) and confirmed three days later by urine glucose (Bayer reagent strips) and blood glucose (citrate buffer, pH 4.5) and confirmed three days later by urine glucose (Bayer reagent strips) and blood glucose (Sigma, St. Louis, MO) to develop a brown precipitate that was easily observed in the whole-mount retinas. Each staining included a number of controls treated identically to the experimental tissue. The first positive control for TUNEL–HRP staining was a section of paraffin-embedded ventral prostate from a castrated rat (large-scale apoptosis occurs in the ventral prostate two days after castration) (28). This was compared to a similar section from an uncastrated adult rat. To provide a negative control, we also evaluated prostate sections from castrated rats by omitting terminal transferase. The second positive control, a whole-mounted ischemic retina, served as a comparison for neuronal apoptosis in whole-mounted retina. Retinal ischemia was induced in normal rats by raising the intraocular pressure of one eye to 100 mmHg for 60 min with saline injected by a peristaltic micro-pump, causing the eye to blanch. This technique causes ganglion cell apoptosis for several days following reperfusion (28–30). TUNEL staining in the ischemic retina was compared to that in the contralateral (non-ischemic) retina. The three negative controls were: (1) sections of apoptotic prostate from which the terminal transferase enzyme was omitted; (2) sections from a normal prostate stained with TUNEL–HRP; and (3) the contralateral (non-ischemic) retina also stained with TUNEL–HRP.

For the colocalization study, TUNEL–FITC was used to stain 10-μm cryostat sections simultaneously with a polyclonal antibody to von Willebrand factor (vWF, 1:500; Dako Corp., Carpinteria, CA). The TUNEL–FITC protocol was followed by an overnight incubation with anti-vWF in 10% goat serum with 0.1% Triton in PBS. The anti-vWF was detected with a rhodamine-conjugated donkey anti–rabbit IgG (1:600; Jackson ImmunoResearch Laboratories, West Grove, PA).

Postmortem eye specimens. Human eyes from anonymous donors were obtained from the National Disease Research Interchange (Philadelphia, PA). The following selection criteria were applied: < 65 yr old, with either insulin-requiring diabetes or no diabetes, no life-support measures, and no chemotherapy. The donors were selected in pairs to be approximately age matched. The eyes were enucleated an average of 5.3 ± 2.5 h after death, and preserved by immersion in normal buffered formalin for 48–72 h and processed for paraffin embedding.

Histology and image analysis. Eyes from 7.5 mo diabetic (n = 6) and aged-matched control rats were analyzed. 16 sections (6 μm) taken from the midpoint of each eye at approximately 40 μm intervals were stained with thionin, a Nissl stain, to identify neuronal cell bodies (24). Images were captured using Optimas software on a PC computer linked to a high resolution video camera (DXC-90MD; Sony) mounted on a microscope (BH-2; Olympus, Melville, NY). Five visual fields were sampled from the posterior portion of each retina using a 40× objective (each visual field representing a 340 μm length of retina). Within each visual field, the thickness of the inner plexiform layer, inner nuclear layer, and entire retina (excluding photoreceptors) was measured by the method of Hughes (25). To compensate for any bias due to skewed sectioning angle (i.e., deviation from 90°), all measurements were expressed relative to the thickness of the choroid in the respective section. The number of ganglion cells was also quantified in each visual field and the total count for the five sampled fields was expressed per 100 μm. Cells were categorized as ganglion cells only if they appeared in the ganglion cell layer and had large, round cell bodies containing positively-stained Nissl substance.

Terminal transferase dUTP nick end labeling (TUNEL). TUNEL was performed with horseradish peroxidase detection (TUNEL–HRP) in whole-mount retinas (26, 27), or with fluorescein detection (TUNEL–FITC) in 10-μm cryostat sections. Isolated retinas were fixed in normal buffered formalin for 10 min at room temperature, and flattened on microscope slides coated with 2% 3-aminopropyltriethoxy silane in humidified boxes, at 4°C overnight, before storing at −20°C. Before TUNEL–HRP staining, the retinas were rehydrated in PBS and fixed in 4% parafomaldehyde for 10 min. The tissue was then dehydrated through graded alcohols, and defatted in xylene overnight to allow better penetration across the inner limiting membrane of the retina (27). The following day, the sections were rehydrated and permeablized with 0.3% Triton for 15 min, and digested with proteinase K (20 μg/ml, Sigma) for 30 min. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min. TUNEL–HRP staining was performed with the horseradish peroxidase ApopTag kit (Oncor Inc., Gaithersburg, MD), following the manufacturer’s instructions. This kit uses terminal transferase to tag single or double strand 3'-OH ends of DNA with digoxigenin-11-dUTP and dATP, which is then recognized with an HRP-bound antidigoxigenin antibody. The HRP was reacted with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Gibco BRL, Gaithersburg, MD) and 0.03% hydrogen peroxide (Sigma, St. Louis, MO) to develop a brown precipitate that was easily observed in the whole-mount retinas. Each staining included a number of controls treated identically to the experimental tissue. The first positive control for TUNEL–HRP staining was a section of paraffin-embedded ventral prostate from a castrated rat (large-scale apoptosis occurs in the ventral prostate two days after castration) (28). This was compared to a similar section from an uncastrated adult rat. To provide a negative control, we also evaluated prostate sections from castrated rats by omitting terminal transferase. The second positive control, a whole-mounted ischemic retina, served as a comparison for neuronal apoptosis in whole-mounted retina. Retinal ischemia was induced in normal rats by raising the intraocular pressure of one eye to 100 mmHg for 60 min with saline injected by a peristaltic micro-pump, causing the eye to blanch. This technique causes ganglion cell apoptosis for several days following reperfusion (28–30). TUNEL staining in the ischemic retina was compared to that in the contralateral (non-ischemic) retina. The three negative controls were: (1) sections of apoptotic prostate from which the terminal transferase enzyme was omitted; (2) sections from a normal prostate stained with TUNEL–HRP; and (3) the contralateral (non-ischemic) retina also stained with TUNEL–HRP.

References

1. Methods

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Data collection and analysis. The percent weight change of rats was calculated as the difference between the weights at STZ injection and sacrifice, divided by the initial weight and multiplied by 100. The blood glucose and percent weight change data were analyzed by Mann-Whitney or Kruskal-Wallis nonparametric tests. Analysis of the morphometry data was by ANOVA or unpaired t-test, where appropriate.

The total number of TUNEL-HRP–positive cells was recorded for each retina in a masked fashion. Each retina was visually scanned with a high power (40×) objective in a serpentine manner to observe the entire retina. The number of TUNEL-HRP–positive cells was expressed per unit area, measured by tracing the outline of each retina viewed with a dissection microscope attached to the image analysis system described above. TUNEL-HRP–positive cell count data was log_{10} transformed to compensate for unequal group variances before analysis of variance. Individual differences between groups were measured using the Newman-Keuls test.

Results

The retina degenerates during diabetes. To determine if atrophy and cell loss occur in the retina during diabetes, the relative thickness of the layers of the retina were measured in diabetic and age-matched control rats. Image analysis of serial sections of paraffin-embedded eyes from rats after 7.5 mo of STZ diabetes was used to measure changes in the thickness of the entire retina, the inner plexiform and nuclear layers, and to quantify the ganglion cell density. To standardize for bias introduced by skewed sectioning angles, all measurements were expressed relative to the thickness of the choroid in each section, which did not vary significantly with diabetes, averaging 101.5±5.7 μm in diabetic rats and 105.7±8.1 μm in controls (t-test, t = 0.47, P > 0.6). The diabetic rats had a significantly reduced weight (percent weight change was 59.5±9.29 in the diabetic group and 154.3±13.47 in controls, n = 6, P < 0.001) and elevated blood glucose (154.3±13.47 mg/dl in diabetic group and 65.3±4.73 mg/dl in controls, P < 0.001). The thickness of the inner plexiform layer was reduced by 22% of control and the inner nuclear layer was reduced by 14% of control, representing a significant reduction (t-test, P < 0.001 in both cases; Fig. 1 A). The total thickness of the retina was also reduced by 13% of control. This reduction was completely accounted for by the reduced thickness in the inner plexiform and nuclear layers; the combined thickness of the outer plexiform and outer nuclear layers did not change (t = 0.775, P > 0.4). The average ganglion cell density in the diabetic rats was 72.8±0.84/100 μm, while that of controls was 80.5±1.19/100 μm (t-test, P < 0.001; Fig. 1 B), a 10% reduction. This reduction in the number of ganglion cell bodies, combined with the overall reduction in the thickness of the inner plexiform layer, indicates that there is a significant ganglion cell loss in the retina after 7.5 months of STZ diabetes. Additionally, the reduced thickness of the inner nuclear layer could be accounted for by the death of neurons and Müller cells. These data demonstrate that retinal degeneration occurs in diabetes and suggest that neural cells die at an accelerated rate compared to age-matched controls.

Retinal cell death occurs by apoptosis during diabetes. Morphometric analysis of sections of long-term diabetic rat eyes suggests that there is an accelerated rate of death of retinal ganglion cells, neurons, and possibly glial cells. To characterize and quantify this cell death, and to determine the time course of the increase, the number of apoptotic nuclei were counted in retinas after 1, 3, 6 and 12 mo of STZ diabetes. Because apoptotic cells are sparsely distributed in other retinal degenerations (22, 23), we stained whole-mount retinas using the TUNEL-HRP method to identify nuclei containing fragmented DNA, a characteristic of cells undergoing apoptosis, and the total number of TUNEL-HRP–positive cells in each retina was expressed per 0.5 cm². This enabled an estimate of the number of apoptotic nuclei in the entire retina, and provided a superior sampling technique compared with TUNEL staining in histological sections.

Table I. Weight Change and Blood Glucose of Rats in the Apoptosis Study

<table>
<thead>
<tr>
<th>Duration of diabetes (mo)</th>
<th>Treatment group</th>
<th>n</th>
<th>% Weight change±SEM</th>
<th>Blood glucose±SEM (mg/dl)</th>
<th>% Glycosylated hb±SEM</th>
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<tbody>
<tr>
<td>1</td>
<td>control</td>
<td>6</td>
<td>26.7±1.58</td>
<td>85.7±14.99</td>
<td>6.2±2.43</td>
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<tr>
<td></td>
<td>diabetic</td>
<td>6</td>
<td>-11.3±4.61†</td>
<td>334.2±42.43‡</td>
<td>14.1±1.05*</td>
</tr>
<tr>
<td>3</td>
<td>control</td>
<td>6</td>
<td>72.2±4.18</td>
<td>68.7±2.45</td>
<td>—</td>
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<tr>
<td></td>
<td>diabetic</td>
<td>6</td>
<td>-7.6±6.38§</td>
<td>328.7±6.9‡</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>control</td>
<td>6</td>
<td>114.7±6.80</td>
<td>62.8±3.70</td>
<td>6.0±0.99</td>
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<tr>
<td></td>
<td>diabetic</td>
<td>6</td>
<td>13.0±8.86</td>
<td>242.5±37.81§</td>
<td>14.7±1.22†</td>
</tr>
<tr>
<td>12</td>
<td>control</td>
<td>4</td>
<td>119.1±13.84</td>
<td>52.8±3.64</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>diabetic</td>
<td>5</td>
<td>27.0±13.45*</td>
<td>201.6±29.06*</td>
<td>—</td>
</tr>
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</table>

Rats were injected with streptozotocin and housed alongside age-matched controls for 1, 3, 6, and 12 months. % Weight change and plasma glucose were measured as described under Methods. Glycosylated hemoglobin values were also measured in blood samples taken at sacrifice in the one and six month groups. The % weight change, plasma glucose and glycosylated hemoglobin values were significantly different from control in all diabetic groups. (†P < 0.05, ‡P < 0.005, Mann-Whitney test).

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All diabetic rats gained significantly less weight than their respective age-matched control groups (Table I). The blood glucose values of the diabetic rats were significantly higher than control values at all four time points \((P < 0.05)\). Glycosylated hemoglobin, which is a measure of chronic hyperglycemia, was also significantly higher than control values in the diabetic groups at both time points measured.

TUNEL-HRP-positive cells were observed in retinas of diabetic rats (Fig. 2), indicated by a brown precipitate staining nuclei. These cells were easily recognized against negligible background staining and appeared identical to TUNEL-HRP-positive cells in both the ischemic retinas and the ventral prostate from castrated rats. In many instances, TUNEL-HRP positive nuclei had a typical apoptotic morphology including pyknosis and nuclear fragmentation (Fig. 2). The vasculature of the retina could also be visualized with normal light field optics and was further resolved by differential interference contrast. The vast majority \((> 90\%)\) of TUNEL-HRP-positive cells were distinct from observable vasculature. TUNEL-HRP-positive cells appeared occasionally in all parts of the retina, mostly as individual cells or as loosely associated groups (Fig. 3) at different depths in the whole mounts, noted by focusing through the tissue.

Diabetic rats had significantly more TUNEL-HRP-positive cells at all four time points than their respective age-matched controls (Fig. 4; 2-way ANOVA with post-hoc New-
First month of diabetes, rats were given subcutaneous insulin implants 3 d after STZ injection, and killed 1 mo later. The retinas were whole-mounted and stained with TUNEL-HRP as described earlier. The uncontrolled diabetic group (no insulin implants) gained significantly less weight ($P < 0.001$) and had higher blood glucose values than the control group ($P < 0.0001$) (Table II). The insulin-treated group had slightly elevated plasma glucose levels compared with controls ($P < 0.05$). Soon after the onset of diabetes, the average weight of the diabetic group became significantly less than that of the control group, while the insulin-treated group gained weight at the same rate.

### Table II. Weight Change and Glycosylated Hemoglobin of Rats in the Insulin Study

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>$n$</th>
<th>% Weight change±SEM</th>
<th>% Glycosylated hb±SEM</th>
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<tr>
<td>Control</td>
<td>12</td>
<td>27.51±0.92</td>
<td>4.03±0.09</td>
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<tr>
<td>Untreated diabetic</td>
<td>12</td>
<td>$-2.51±2.35^{**}$</td>
<td>$12.04±0.47^{1}$</td>
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<tr>
<td>Insulin-treated diabetic</td>
<td>10</td>
<td>26.88±2.07</td>
<td>6.93±0.46*</td>
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</table>

The % weight change of the insulin-treated diabetic group was less than the other groups ($^{**}P < 0.0001$) while that of the insulin-treated group was significantly different from both the other groups ($^{*}P < 0.05$).

Insulin reduces apoptosis in the retina. To determine the effect of insulin on diabetes-induced retinal cell death during the first month of diabetes, rats were given subcutaneous insulin

Apoptotic cells are distinct from the vasculature. Quantification by TUNEL staining found a large increase in apoptosis in diabetic rat retinas. The whole-mount TUNEL technique, however, is incompatible with immunohistochemical identification of cell types because of the extensive exposure to xylene. Morphometric measurements indicated that the number of surviving retinal ganglion cells is significantly reduced over a 7.5 mo period of diabetes. Since apoptosis of vascular cells is established in diabetes (3), we sought to confirm that retinal apoptosis is not restricted to vascular endothelial cells.

TUNEL-FITC labeling was carried out in cryostat sections of retina simultaneously with immunohistochemical labeling of the vascular endothelial cell antigen, vWF. Tissue from both diabetic and control rats had similar degrees of positive vWF immunoreactivity and a small number of sections had positive TUNEL-FITC labeling (Fig. 5). TUNEL-FITC labeling was observed in the ganglion cell layer. These cells had large cell bodies characteristic of ganglion cells, and did not colocalize with vWF immunoreactivity. These data confirm that nonvascular cells, most likely ganglion cells, become apoptotic in the diabetic rat retina.

Insulin reduces apoptosis in the retina. To determine the effect of insulin on diabetes-induced retinal cell death during the first month of diabetes, rats were given subcutaneous insulin

Figure 4. Apoptosis is increased in diabetic rat retinas. Rats were made diabetic by intravenous STZ injection and housed for between 1 and 12 months (black circles) alongside age-matched controls (white circles). Whole-mounted retinas were stained for apoptotic cells by the TUNEL-HRP method and the total number of positive cells was counted in each retina. The number of positive cells was expressed per 0.5 cm². The diabetic rats had significantly more apoptotic cells compared to normal age-matched controls at all four time points ($^{*}P < 0.01$, $^{**}P < 0.001$, ANOVA with Newmann-Keuls test).

Figure 5. TUNEL positive cells are not vascular. Cryostat sections of rat retina were double labeled for the endothelial cell-specific antigen, von Willebrand factor (vWF, rhodamine label) and with TUNEL (fluorescein label). The ganglion cell layer of the retina contains a number of TUNEL-positive (green) cells that do not colocalize with vWF (orange-red) (Double exposure using fluorescein and rhodamine filter sets). Digital image reproduced from original 35mm transparency, ×200). RGC, retinal ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelial layer.
The average blood glucose in the diabetic group was high from the onset of diabetes, but that of the insulin-treated group gradually reduced to control levels over a 12-d period (Fig. 6B). As the insulin-treated rats gained weight, the number of implants given to each was increased to deliver the appropriate dose of insulin to maintain normoglycemia. The increase in the number of TUNEL-HRP–positive cells occurring in untreated diabetic rat retinas was consistent with our earlier findings (Fig. 7; P < 0.01, compared to respective control group). The number of TUNEL-HRP–positive cells in the retinas of the insulin-treated rats was significantly less than in the diabetic group (P < 0.05), and not significantly greater than the control group. These data suggest that early insulin replacement reduces the number of apoptotic cells in the retina of the diabetic rat. The effect of insulin suggests that the increase in TUNEL-HRP reactivity is not due to a confounding effect of STZ injection, but is a direct consequence of diabetes.

**Apoptosis is increased in the retinas of humans with diabetes.** To determine if retinal apoptosis occurs to a similar degree in the retinas of humans with diabetes, we examined five postmortem human retinas from donors with and without diabetes. The retinas were divided into quadrants, flat-mounted, and labeled using the TUNEL-HRP protocol in the same way as the rat retinas in earlier experiments. Adhering retinal pigment epithelial cells were mostly washed off the specimens before flat-mounting, and the few remaining pigmented cells were easily identified by their position below the plane of the retina, and were not confused with TUNEL-HRP–positive cells. Table III summarizes the clinical details of each donor along with the number of apoptotic cells per cm². Subjects 1, 2, and 3 had no history of diabetes, while Subjects 4 and 5 had confirmed histories of diabetes for 6 and 30 yr, respectively, and both used insulin. Subject 5 also had a confirmed history of diabetic retinopathy. The vasculature of each retina was examined with both light field and differential interference contrast optics for indications of abnormalities. The retina of Subject 5 contained many microaneurysms typical of diabetic retinopathy. The retina of Subject 3 also contained an area

![Figure 6. Insulin implants reduce blood glucose and stabilize weight loss in diabetic rats. Streptozotocin diabetic rats were given subcutaneous insulin implants for 1 month of diabetes. (A) Diabetic rats lost weight (black circles) while age-matched controls (white circles) and diabetic rats with insulin implants (black squares) both gained weight at the same rate. (B) Plasma glucose levels were elevated in the diabetic rats and normal in controls. The levels in diabetic rats with insulin implants gradually decreased to control levels over an initial 12-d period and remained close to normal for the duration of the experiment.](image1)

![Figure 7. Insulin reduces retinal apoptosis in experimental diabetes. Whole-mount retinas were stained with TUNEL-HRP after 1 month of untreated diabetes (striped bar) or diabetes with insulin implants (crosshatched bar) and the number of TUNEL-HRP–positive nuclei compared to that of age-matched controls (white bar). The diabetic rats had significantly more TUNEL-HRP–positive cells than controls (*P < 0.001) and the insulin treated group (P < 0.05).](image2)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diabetes duration (yr)</th>
<th>Retinal pathology</th>
<th>Cause of death</th>
<th>TUNEL-HRP positive cells/0.5 cm²</th>
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<tr>
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<td>48.4</td>
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<tr>
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<td>30</td>
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<td>MI</td>
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</tr>
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</table>

Table III. Clinical Characteristics of Eye Donors and Number of TUNEL-HRP Positive Cells in Whole-Mounted Retinas

Postmortem human retinas were examined for vascular abnormalities and stained for TUNEL-HRP positive cells as described. CVA, cerebrovascular accident; MI, myocardial infarction; CLD, chronic lung disease; CHF, congestive heart failure; NPDR, non-proliferative diabetic retinopathy; BVO, branch vein occlusion.
characteristic of branch vein occlusion, in which vasculature
was not clearly visible. No other vascular abnormalities
were observed in the other specimens. Many TUNEL-HRP–
positive cells had typical pyknotic morphology and were mostly
distributed as single cells (Fig. 2). The subjects without diabe-
tes had an average TUNEL-HRP–positive cell count of
70.9±21.9 cells/cm² (58±14.8 cells/cm², excluding Subject 3),
while the subjects with diabetes had an average of 145±20.8
cells/cm². These data indicate that retinal apoptosis is a gen-
eralized response of retinal neural cells to diabetes and is not a
specific feature of STZ-induced diabetes in rats. Furthermore,
TUNEL-HRP–positive cells were observed away from micro-
aneurysms in a human retina with diabetic retinopathy, and
are therefore not limited to areas with retinal vascular lesions.

Discussion

This study reports that both experimental diabetes in rats and
diabetes mellitus in humans are accompanied by increased
apoptosis of retinal neural cells. The increase in the frequency
of apoptosis occurred after only 1 mo of experimental diabetes
in rats, and a similar increase was noted in a human retina after
six years of diabetes. The significant reduction of retinal gan-
glion cells and the reduction in the thickness of the inner plexi-
form and nuclear layers after 7.5 months of STZ diabetes sug-
gest that the apoptotic cells are likely to include ganglion cells
and other neurons. The histological location of TUNEL-posi-
tive cells and lack of colocalization with von Willebrand factor
further suggest that neuronal and/or glial cell apoptosis occurs
in the retina in diabetes. Viewed in the light of previous obser-
vations of neuronal cell death in diabetes (7–9), these data
strongly suggest that neuronal cells begin to die soon after the
onset of experimental diabetes in rats. They also confirm an-
other brief observation of apoptosis in retinas of humans with
diabetes, which were included in the control group of a study
reporting apoptosis in open angle glaucoma (23). Therefore,
neurodegeneration should be considered a component of dia-
betic retinopathy, as was suggested by both Wolfer and Blood-
worth (7, 8). Indeed, focal areas of retinal thinning have also
been described in patients with diabetic retinopathy (32), and
nerve fiber layer defects occur in patients with minimal or no
vascular lesions (33).

Accelerated apoptosis of retinal vascular cells has been
measured in trypsin-digested retinas from both rats and hu-
mans with diabetes (3), but the magnitude of apoptosis re-
ported here indicates that most of the apoptotic cells in the ret-
ina are not endothelial cells or pericytes. Since quenching of
endogenous peroxidase greatly reduced background staining
associated with the vasculature and erythrocytes, TUNEL-
HRP–positive cells were not confused with components of the
blood. Furthermore, the increase in TUNEL-HRP staining is
unlikely to be due to ischemic necrosis since it occurs after
only one month of experimental diabetes in rats, before the
onset of any known vascular occlusion. Neural cell death due
to increased vascular permeability cannot be ruled out, how-
ever, since some increase in permeability can occur after one
month of experimental diabetes in rats (34).

This study quantified the number of apoptotic cells in dia-
betic rat and human retinas by surveying whole-mount retinas.
Other studies have observed some TUNEL-positive labeling
in cryostat sections of diabetic rat eyes (35) and human retinas
(23). Judging from the total number of apoptotic cells counted
in the present study, it is unlikely that TUNEL staining of his-
tological sections can be used to accurately quantify apoptosis
in the diabetic retina, since a very large number of sections
would have to be stained to make a reasonable sample. The
section containing TUNEL-positive nuclei illustrated in Figure
5 was one of many radial sections that were found to contain
no TUNEL-positive staining. This pattern of staining of radial
sections is in line with the distribution of TUNEL-positive nu-
clei illustrated in Figure 3. It is clear that apoptosis also occurs
in the normal retina, so in order to demonstrate an increase in
apoptosis above baseline levels we sampled the entire retina.

Could the frequency of apoptosis explain the reduction in
retinal thickness? The rat pup retina has 24.8±0.96 million
cells at postnatal day 8, at which time the percentage of mitotic
cells is negligible (36); so a reasonable estimate of the total
number of retinal cells in the adult rats used here is ~20 mil-
V

lion. Estimating the length of time that cells contain frag-
mented DNA as 6–12 h (37), the number of apoptotic cells
measured here predicts a total loss of retinal cells of 1–10%
over a 12 mo period. This is consistent with the morphometric
changes we measured in the rat retinas after 7.5 months of dia-
betes. This degree of neural cell loss would not be detected
clinically since the neural retina is normally transparent. The
gradual nature of the cell death is consistent with the long la-
tent period between the onset of diabetes and detectable dia-
betic retinopathy.

The increase in apoptotic cells in diabetic rats was mirrored
in postmortem retinas from humans with diabetes. These data
confirm that retinal neural cell death is not specific to STZ dia-
betes in rats. It is important to note that the number of apop-
totic cells in both human retinas was about the same, but only
one had detectable vascular lesions. The other donor was
known to have had diabetes for only six years, and clinically
evident vascular abnormalities do not usually develop during
this short a duration of diabetes in humans. Therefore, these
data suggest that retinal apoptosis is not a late consequence of
vascular lesions.

Insulin delivered during the first month of diabetes re-
cued the number of apoptotic cells in the retina. These data
suggest that retinal apoptosis in diabetes can be reversed by in-
sulin therapy. Insulin may act by reversing some aspect of the
physiology of diabetes (such as hyperglycemia). Indeed, galac-
tosemic rats, which are hyperglycemic but not insulin deficient,
also have increased vascular cell apoptosis (3). There is also
extensive evidence that insulin can act as a trophic factor that
affects cell survival in the central nervous system. Insulin re-
duces neuronal cell death in cerebral ischemia independently
of blood glucose levels (38). Insulin regulates a number of fac-
tors associated with apoptosis, including neuronal intracellular
calcium levels (39), calretinin and calbindin (40). Furthermore,
inulin is a required survival factor in the central nervous sys-
tem. At physiological concentrations, insulin rescues cultured
optic nerve oligodendrocytes from death induced by trophic
factor withdrawal (41), and is necessary for survival of retinal
ganglion cells in culture (42). Insulin also rescues retinal vascu-
lar endothelial cells when apoptosis is induced by high glucose
media (43). Regardless of the mechanism, these data strongly
suggest that retinal apoptosis is reduced by exogenous insulin.

Secondary toxic factors may also contribute to retinal apop-
tosis in diabetes. Changes in glutamate metabolism occur in
short-term experimental diabetes (6), implying that glial cells
in the diabetic retina provide less protection from extracellular

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glutamate accumulation. An increase in the amount of unmetabolized glutamate in the retina may cause glutamate toxicity, which may in turn lead to neuronal apoptosis. Furthermore, retinal glial cells express more glutamate-fibrillary acidic protein in early experimental diabetes (6) and in human diabetes (5), indicating a reactive state typical of central nervous system injury.

An increase in neural cell apoptosis could also contribute to microangiopathy because the apoptotic cells may include glia. Glial cells induce barrier properties in brain (44) and tight junction protein expression in retinal vascular endothelial cells (45). If retinal glial cells die, this could lead to a loss of endothelial cell barrier properties. Thus, retinal astrocyte or Müller cell death could impair barrier function. Indeed, other results from this laboratory show that the tight junction protein, occludin, is reduced in rat retinas after short-term STZ diabetes (Antonetti et al., manuscript submitted for publication).

This study addresses the two questions posed in the introduction. The data suggest that: (1) apoptosis begins soon after the onset of diabetes and continues at a relatively constant rate; and (2) the apoptotic cells include ganglion cells and other neural cells. Removal of insulin by STZ gives rise to neural cell death in the retina, but it is not yet established whether this relationship is a direct one or if the loss of insulin gives rise to apoptosis by an indirect mechanism, such as hyperglycemia. In light of these data, we suggest that diabetic retinopathy should be viewed as a disease that includes retinal neurodegeneration as well as vascular pathologies. Further attempts to understand and treat this disease must include a consideration of the roles of all three cellular elements (neurons, glial and vascular cells), and how they interact under the physiological constraints of diabetes.

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