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Abstract

Diabetic retinopathy is one of the most common causes of vision loss in many countries. This disease has been characterized as primarily one of the inner blood-retinal barrier, because of the well-established increase in vascular permeability, microaneurysms and angiogenesis. Growing evidence suggests, however, that the mechanism of this disease is more complex and involves many, if not all of the different types of cells in the retina. The results of numerous studies demonstrate that apoptosis is a critical part of the pathology of diabetic retinopathy, which on first inspection seems paradoxical since the disease
culminates in the proliferation of vascular endothelial cells. Data from experimental animal models and postmortem human tissue suggest, however, that apoptosis also occurs in the neural cells of the retina. Apoptosis of neural cells may occur soon after the onset of diabetes, leading to chronic degeneration of the retina. This gradual disappearance of neurons (and possibly glial cells) may occur as a consequence of the loss of blood-retinal barrier function that leads to vascular permeability and macular edema. Alternatively, neural apoptosis may be a direct result of the metabolic changes in diabetes. Here the history of this notion and the major experimental findings that established apoptosis as a feature of diabetic retinopathy will be examined. Also, the subtypes of retinal cells lost by apoptosis and the possible triggering mechanisms will be considered.

Introduction

Diabetic retinopathy: A leading cause of vision loss

Diabetic retinopathy is a common complication of diabetes that causes loss of vision in many people. It is the leading cause of legal blindness in working-age adults in the western world [1]. The disease is clinically recognized by alterations in the retinal vasculature that makes up the inner blood-retinal barrier and particularly by an increase in vascular permeability leading to macular edema, in which the central part of the retina swells due to accumulating fluid from abnormally permeable blood vessels. Other vascular abnormalities usually appear later and include vascular swellings called microaneurysms, and angiogenesis, in which new microvessels proliferate and tend to be permeable and fragile. In the proliferative stage of the disease there can be acute hemorrhage of new vessels as well as formation of vascularized intravitreal membranes accompanied by retinal detachment. Profound vision loss can occur in diabetic retinopathy, associated with acute hemorrhage and ischemia during the later stages of the disease, but macular edema tends to occur earlier in the disease and is closely correlated with loss of visual acuity [2, 3].

The alarming increase in the prevalence of diabetes is one of the major challenges to modern health care. In the United States it has been estimated that about 18 million people have diabetes, accounting for more than 6% of the population (American Diabetes Association national fact sheet). After 15 years of diabetes, between 77.8 and 97.8% of people will have some degree of diabetic retinopathy, depending on the initial age of onset [4, 5]. This makes diabetic retinopathy the leading cause of new cases of legal blindness in adults between the ages of 20 and 74 years [6].

The vision loss due to diabetic retinopathy has some specific features including loss of night vision [7, 8], and contrast sensitivity [9-12], and loss of
sensitivity to contrast between certain colors such as blue and yellow [13]. Subtle deficits in vision function may occur very soon after the onset of diabetes, before clinically significant vascular abnormalities can be detected [9, 14]. The electrophysiological signature of the retina (electroretinogram) is also altered in specific ways that suggest a loss of neural function [15, 16]. Again, the electrophysiological changes appear to be early events in the time course of diabetic retinopathy [17, 18]. These observations suggest that the function of the neural retina is compromised by diabetes even before clinically detectable changes occur in the vasculature, and that the vision loss is probably due to damage at the level of photoreceptors or neurons.

The only established treatment for diabetic retinopathy, apart from tight control of blood sugar, as recommended by the Diabetes Control and Complications Trial [19, 20], is laser photocoagulation, which halts the progress of the disease in about 50% of cases but must be repeated for many patients, and can lead to reduction in the peripheral visual field [21, 22]. In order to develop improved pharmacological approaches to treating the disease, research has focused on the mechanisms of retinal degeneration. In the last few years some of this work has identified retinal cell apoptosis as a key event associated with diabetes.

**Degeneration as an intrinsic component of diabetic retinopathy**

It is likely that the first observations of apoptosis in diabetic retinopathy were made even before the word "apoptosis" was introduced to the scientific vocabulary. Early histopathology studies noted signs of cell loss and degeneration throughout the retina of postmortem pathology specimens. A postmortem histological study of eight eyes from humans with diabetes identified a number of characteristics including vascular pathology and degeneration of neurons, including swollen and "hyalinized" cells, which are rounded acidophilic bodies thought to be involved in degeneration. A predominance of these degenerative features was found in the retinal ganglion cell layer, accompanied by loss of nerve fibers [23]. In a more extensive study, the histology of postmortem eyes from 157 people with diabetes was compared to 138 eyes from non-diabetic donors [24]. The study noted a loss of retinal ganglion cells and other neurons, and appearance of "pyknosis with hyalinization." These early studies were the first to identify signs of cell loss in the retina as a consequence of diabetes, and even went as far as to suggest that this cellular degeneration was the most likely reason for the loss of vision encountered by persons with diabetes. Other studies at that time concentrated specifically on pathological characteristics in the retinal vasculature. The entire microvascular plexus was revealed using a trypsinization technique in which the neural parenchyma is removed by enzymatic digestion after tissue fixation, exposing the intricate structure of the vasculature, which is then carefully mounted flat
onto a glass slide and stained for microscopic examination. Using the trypsin digest technique it was noted that mural cells, also called pericytes, were missing in regions of abnormal vasculature such as microaneurysms. It was suggested that the presence of these cells was required to keep abnormal growth of the endothelial cells in check, and that loss of pericytes was the primary lesion responsible for vascular proliferation in diabetic retinopathy [25]. The function of pericytes is still unclear but since they share common features with vascular smooth muscle cells they may have contractile properties that play a role in regulating capillary blood flow [26].

While the clinical consequence of retinal cell loss in diabetes was quite apparent, the concept of apoptosis as an active mechanism of degeneration was not established in cell biology until the early 1970's [27]. In the absence of strong experimental data on degeneration during the early stages of diabetic retinopathy it was reasonable to assume that apoptosis was a relatively late consequence of ischemia induced by poor perfusion of angiogenic vessels. However, more recent findings have suggested that retinal cell apoptosis begins much sooner after the onset of diabetes than was first thought, and may play an integral role in the early stages of vision loss.

**Apoptosis of vascular cells of the retina**

Despite the fact that vascular proliferation occurs late in diabetic retinopathy, it has been suggested that apoptosis of endothelial cells is increased during the earlier stages of the disease. This concept was explored by quantification of vascular apoptosis using terminal dUTP nick end labeling (TUNEL) in both human and rat retinas, in which the histology of the vasculature was revealed after trypsin digestion of the retinal parenchyma [28]. In this study the postmortem retinas of seven human donors, who had diabetes for an average of 9±4 years, a relatively short duration of diabetes in terms of the time-course of retinopathy, were compared to nine retinas from non-diabetic donors. An average of 13±12 TUNEL-positive cells were found in the vasculature from diabetic donors while only 1.3±1.4 cells were found in the non-diabetic donors. This result was reflected in trypsin-digested retinas from five rats, made diabetic with streptozotocin (STZ), a toxin that renders the rat permanently unable to manufacture insulin by inducing apoptosis in the pancreatic beta cells after a single injection [29, 30]. The rats were STZ-diabetic for 31 weeks, although the magnitude of the increase in apoptotic cells was not as great as in the vasculature from human retinas. On average, 9±6 TUNEL-positive cells per retina were found in the vasculature from the STZ-diabetic rats while 0.4±0.9 were counted in the control samples. This study was the first to report quantification of the number of apoptotic cells in the entire vasculature of each retina. The data suggested that there was a small but significant increase in vascular cell apoptosis. These data supplied a mechanism
for the appearance of acellular capillaries, a characteristic in trypsin digest specimens from diabetic rats, which is thought to model the vascular lesions found in human diabetic retinopathy [31].

Other studies confirmed that apoptosis could be detected by TUNEL in small numbers of cells in retinal trypsin digests. There was an increase in apoptosis in the retinal vasculature from rats after 6-8 months of diabetes induced by alloxan, a drug similar to STZ, followed by increased pericyte ghosts (pockets within the basement membrane that appear to have once contained a pericyte) and capillary dropout after 18 months of diabetes [32]. The study reported a total of $0.8 \pm 1.3$ TUNEL-positive cells per control retina, and $8.8 \pm 6.5$ in the alloxan-diabetic retinas. The antioxidant drug, aminoguanidine, reduced apoptosis in the diabetic rat retinal vasculature to control levels. Similar results were obtained in another study of trypsin-digest retinas from STZ-diabetic rats after hyperglycemia for 11 months [33]. There were $4.1 \pm 2.2$ TUNEL-positive apoptotic cells in the vasculature from STZ-diabetic rats, compared to $1.6 \pm 1.0$ positive cells in controls. A group given the antioxidant, alpha-lipoic acid, in their food for the entire duration of diabetes had $2.1 \pm 1.4$ TUNEL-positive cells, suggesting that oxidative stress may be part of the trigger mechanism for the vascular apoptosis. The number of acellular capillaries was also reduced by a similar degree by alpha-lipoic acid.

Observations of apoptosis and the vascular abnormalities in animal models of diabetic retinopathy led to studies of apoptosis of cultured vascular endothelial cells. The effects of glucose on cell division and apoptosis have been tested extensively in human umbilical vein endothelial cells. It is thought that this cell culture model is equivalent to retinal endothelial cells because of the similar barrier properties in the umbilical vein. The growth rate of these cells was slowed by 20 mM glucose, a concentration that reflects hyperglycemia, and the number of cells in S phase was significantly greater than control cultures grown in 5 mM glucose or the equivalent concentration of mannitol. The higher concentrations of glucose also increased lactate dehydrogenase in the media, indicating cell damage [34]. In similar umbilical vein endothelial cultures, growth in 30 mM glucose media increased the rate of alkali-induced DNA unwinding, suggesting a greater number of DNA strand breaks. This was confirmed by increases in DNA fragmentation in cultures grown with 30 mM glucose [35]. Similar media conditions also increased the amount of hydroxy-urea-resistant thymidine incorporation, suggesting that elevating extracellular glucose increases DNA repair activity [36].

Further support for glucose-induced apoptosis was obtained by studying the "executioner" enzyme, caspase-3. Caspase-3 activity was increased by 50% in retinal capillary endothelial cells by incubating in 25 mM glucose media, and could be reduced by antioxidants [37]. Other early markers of apoptosis accompanied the increase in caspase-3 activity. When retinal endothelia and
pericytes were cultured for 10 days in 20 mM glucose media the amount of the pro-apoptosis protein, BAX, associated with mitochondria was increased, as was the amount of cytoplasmic cytochrome C, suggesting that protracted periods of elevated extracellular glucose induced mitochondrial permeability transition in vascular cells [38].

As well as loss of vascular endothelial cells, apoptosis of vascular pericytes has been implicated in diabetic retinopathy. In observations of the vascular structure of postmortem retinas it was noted that pericytes were absent from blood vessels that still contained intact endothelial cells and that microaneurysms tended not to contain pericytes, suggesting that their loss permits uncontrolled proliferation of endothelial cells [25]. Pericyte "drop-out" has been used as an index of diabetic retinopathy in animal models [39, 40].

Given the contractile properties of these cells it is conceivable that their loss could alter the way blood flow is regulated in capillaries of the retina. Culturing pericytes in elevated concentrations of glucose for 5 weeks increased the expression of BAX, as well as annexin V binding, indicating that the early stages of apoptosis had been triggered [41]. High glucose also increased the nuclear DNA binding of NF-kappaB. Furthermore, inhibition of this factor blocked tumor necrosis factor (TNF) and BAX expression, and reduced apoptosis measured by cytoplasmic nucleosomal DNA in cultured retinal pericytes [42]. Interestingly, this study obtained conflicting results with retinal endothelial cells compared to pericytes. One week of culture in high glucose media did not increase NF-kappaB activation or apoptosis in vascular endothelial cells, suggesting that vascular endothelial cells may be more resistant to the induction of apoptosis by high glucose compared to pericytes.

While much has been learned about apoptosis of vascular endothelial cells it is unclear if the modest increase in apoptosis is related to other functional consequences of diabetes, such as loss of blood-retinal barrier integrity. A key component in the early stages of diabetic retinopathy is macular edema, due to increased permeability of the blood-retinal barrier, which is closely correlated with vision loss [1-3]. An increase in retinal vascular permeability is a well documented consequence of diabetes in both humans and animal models, which appears to precede vascular proliferation [43-49]. However, it is not clear if there is a link between vascular cell apoptosis and increases in vascular permeability. The concept that loss of an endothelial cell leaves a transient hole in the vessel wall seems simplistic, since occasional losses in the vasculature of control animals, which are likely to occur during the normal turnover of cells does not appear to increase vascular permeability. Furthermore, a study using STZ-rats to identify the most permeable regions of blood vessels by in situ fixation of fluorescent concanavalin A showed that permeability does not occur at isolated cells or capillaries, but occurs in many vessels simultaneously, first in the larger superficial vessels and then
progressing to the capillaries of the outer plexiform layer within two months of the onset of diabetes [48]. It seems more likely therefore, that vascular permeability increases as a consequence of regulatory changes in tight junction proteins within a broad population of endothelial cells [49].

A more established mechanism for the increases in vascular permeability is through regulation of tight junction proteins by phosphorylation, degradation, and translocation within the endothelial cell. These modifications of tight junctions are closely correlated with increases in paracellular permeability, but it is unlikely that they occur as a contributing cause of apoptosis since down-regulation of tight junction proteins in cultured retinal endothelial cells by treatment with factors that increase permeability, such as histamine or vascular endothelial growth factor (VEGF), does not appear to increase apoptosis [48-53]. VEGF is a potent permeability-inducing factor known to be upregulated in the retina by diabetes in both humans and animal models [54-59]. The increase in VEGF found in both animal models and humans with diabetes is likely to be at least part of the mechanism of vascular permeability and endothelial cell proliferation [54, 60]. This permeabilizing agent also appears to be a survival factor for cells of the retinal vasculature. Regression of blood vessels in the neonatal retina in response to hyperoxia was preceded by reduction in the expression of VEGF, and exogenous VEGF given at the onset of hyperoxia blocked the endothelial cell apoptosis required for vessel regression [61]. When studied in cell culture, VEGF also protected endothelial cells from apoptosis induced by TNF-alpha [62]. The protective effect of VEGF on endothelial cells is likely to be mediated by increases in the expression of anti-apoptotic proteins such as BCL-2 and A1, which were upregulated in human umbilical vein endothelial cells treated with VEGF [63].

It appears that the survival effects of VEGF are not limited to endothelial cells, but may also operate in neurons. VEGF can act as a mitogen for neurons, at least in the peripheral nervous system, because it causes outgrowth of axons from cervical ganglion and dorsal root ganglion cells in primary cultures [64]. VEGF rescued both cerebellar granule neurons and immortalized hippocampal neurons from apoptosis induced by hypoxic-ischemia, probably by activating the PI-3 kinase/Akt-signaling pathway [65]. The neuroprotective effect of VEGF may explain neuroprotection by hypoxic preconditioning, a paradoxical phenomenon where brief periods of mild hypoxia tend to reduce the amount of apoptosis caused by a subsequent prolonged period of hypoxia. In a study using an in vitro model of hypoxic preconditioning, induction of the protective effect was dependent on protein synthesis, and VEGF antibodies blocked the preconditioning effect, suggesting that mild hypoxia may trigger the synthesis of VEGF, which becomes protective during the extended hypoxic insult [66]. The mechanism of the protective action of VEGF may be, at least in part, through increased expression of survival factors BCL-2 and A1 [63].
Furthermore, VEGF protected cultured hippocampal neurons from apoptosis induced by N-methyl-D-aspartate toxicity, acting through a PI-3 kinase-dependent mechanism, although it did not reduce apoptosis caused by staurosporin [67]. The cytoprotective actions of VEGF on endothelial cells and neurons suggest the interesting possibility that VEGF expression in diabetes may occur to reduce damage to the retina during diabetes. The accompanying effect of VEGF to increase vascular permeability in the retina is paradoxical in this context, because loss of the blood-retinal barrier integrity is generally considered to be detrimental.

In summary, several human and animal studies have established modest increases in vascular cell apoptosis in diabetes but how this impacts retinal function is not yet clear. There is no established mechanism for how vascular cell apoptosis leads to vision loss in diabetic retinopathy. The observations of vascular apoptosis also appear incongruous in the light of the well-established permeabilizing and pro-survival effects of VEGF. One possible explanation for the presence of VEGF is that it is upregulated in response to stress in order to prevent apoptosis in cells. At this stage it is not clear if there is a causal relationship between apoptosis of vascular cells and vascular permeability in diabetic retinopathy, and it seems most likely that they are not related because they appear at different times after the onset of diabetes. It is also unlikely that apoptosis of a small number of vascular cells can give rise to the widespread retinal vascular permeability that occurs in diabetes. It does, however, seem reasonable to suggest that apoptosis of cells in the retinal network that transduces the visual signal from photoreceptors to brain, would directly impair vision. The next section deals with the possibility that neurons and photoreceptors undergo apoptosis, leading to chronic retinal degeneration in diabetes.

**Diabetes and apoptosis of retinal neurons**

The trypsin-digest method is an excellent way to isolate and study the vascular cells of the retina. Other work on retinal apoptosis in diabetes examined the entire retina, including the neurons, photoreceptors and glial cells. An initial study suggesting that diabetes caused apoptosis in neurons used TUNEL in histological sections of retinas of STZ-diabetic rats. Apoptotic nuclei were detected in the inner retina, where retinal ganglion cells are localized [68]. Apoptosis was reduced by systemic administration of nerve growth factor, suggesting that neurons were the primary cells affected. TUNEL-positive nuclei were also noted in a small number of histological sections from postmortem retinas of people with diabetic retinopathy, which served as a control group for a study of apoptosis in glaucoma [69]. These studies suggested that apoptosis occurred in retinas of both animals and humans with diabetes. The data were not quantitative, however, because of the
relatively low frequency of TUNEL-positive cells found in radial histological sections, so accurate estimation of the total number of apoptotic cells was not possible.

The initial observations of histological sections of retinas from STZ-diabetic rats and postmortem human tissue suggested that TUNEL positive and pyknotic nuclei in the inner retina were relatively sporadic. Further investigation required more complete quantification of the number of apoptotic cells. To quantify apoptosis in the whole retina, the *in situ* TUNEL assay was adapted to label intact retinas that had been dissected, fixed and flat-mounted onto microscope slides [70]. Using this technique TUNEL-positive cells in retinas of STZ-diabetic and control rats were counted by visually scanning entire retinas with a light microscope and high magnification objective [71]. The total number of positive nuclei in each retina was expressed per unit area of tissue, measured by image analysis of each retina photographed though a dissection microscope, to standardize the data by expressing the cell count per unit area of retina. The data showed that retinal apoptosis was elevated in rat retinas after 1, 3, 6 and 12 months of STZ-diabetes compared to those of age-matched controls. The number of positive cells was about the same at each time point, suggesting that cell loss occurred at a relatively constant rate. The average number of TUNEL-positive cells in the retinas of age-matched control rats was around 20, while in the STZ-diabetic rat retinas it was between 60 and 120 cells. The spatial distribution of TUNEL-positive cells in whole-mount retinas tended to be random. Positive cells usually appeared as isolated individuals rather than in clusters, and were not limited to either the peripheral or central regions. The 3- to 6-fold increase in TUNEL-positive nuclei was statistically significant, but still represented a small number of cells relative to the total number in a rat retina. Similar results, however, were found in another study of STZ-diabetic rats, using the same technique of applying TUNEL to whole retinas [72]. Interestingly an increase in intraocular pressure further elevated the number of TUNEL-positive cells in diabetic rats. Taken together, these data suggested that retinal cell apoptosis is a chronic and possibly random event in diabetes. The fact that significant apoptosis can be measured in whole retinas within the first month of STZ-diabetes implies that this is a direct result of diabetes and not a consequence of other degenerative changes. If neurons are among the cells undergoing apoptosis then there will be a cumulative loss of cells leading to chronic neurodegeneration.

The morphology of rat retinas was examined to determine if the slow rate of cell loss had significant impact on the overall number of cells remaining in the retina after a longer duration of diabetes. Paraffin embedded sections of eyes from STZ-rats after 30 weeks of diabetes were examined for changes in thickness, and the number of large cell bodies in the retinal ganglion cell layer were counted to determine if the cumulative cell loss would result in
significant differences in the total number of remaining cells in diabetic and control retinas [71]. There was a significant reduction in the thickness of the cell layers in the inner retina and a loss of the number of cell bodies in the retinal ganglion cell layer. The total number of cell bodies in the retinal ganglion cell layer was reduced by 10% after 7.5 months of diabetes. This was accompanied by a 22% reduction in the thickness of the inner plexiform layer, and a 14% loss in the thickness of the inner nuclear layer. There was no change in the dimensions of the outer nuclear layer, suggesting that more cells were lost from the inner retina than from the outer. This study attempted to determine something about the nature of the cells undergoing apoptosis by pairing TUNEL with immunohistochemistry for von Willebrand factor, which is often used as a marker of vascular endothelial cells [73]. It was found that at least in the small number of radial sections sampled, TUNEL labeling did not localize with blood vessels, suggesting that the cells undergoing apoptosis were more likely to be neurons or glia than pericytes and endothelial cells.

More recently, similar measurements were made in the retinas of Ins2Akita mice, a spontaneously diabetic strain that develops significant hyperglycemia around 4-5 weeks after birth, due to a point mutation on the second insulin gene, which causes degeneration of pancreatic beta cells [74, 75]. The number of apoptotic cells in mice that had been diabetic for 4 weeks was measured in whole retinas by counting the number of cells with positive immunoreactivity for active caspase-3 [76]. There was an average of 48±4.09 caspase-3 positive cells/0.5 cm² in the control retinas, compared to 109.4±16.46 positive cells/0.5 cm² in the diabetic mice. Morphometry was again used to measure the thickness of the different layers of the retina in mice that had been diabetic for 22 weeks. There was a 16.7% reduction in the thickness of the central part of the inner plexiform layer and a 27% reduction in the peripheral part of this layer. There was also a 15.6% reduction in the thickness of the peripheral inner nuclear layer. Interestingly there was no statistically significant reduction in the thickness of the central retina, suggesting that at least in mice, loss of neurons may occur more rapidly in the peripheral retina. The number of cell bodies in the retinal ganglion cell layer was reduced by 23.4% compared to the littermate controls. Other studies have also reported loss of thickness in retinal cell layers due to experimental diabetes. In retinas of Sprague-Dawley rats there was a 10% reduction in the thickness of the inner plexiform layer after only one month of STZ-diabetes. In contrast the reduction in the inner plexiform layer of Brown-Norway rats with a similar duration of STZ-diabetes was nearly 16% [77]. Even greater losses of thickness in the inner and outer retina have been reported in other mouse models of diabetes, suggesting that neurodegeneration is not limited to the rat STZ model. A loss of between 20 and 25% of cells in the retinal ganglion cell layer was found in radial sections of mouse retinas after 14 weeks of STZ-diabetes [78]. The thickness of the
entire retina was also significantly decreased, suggesting an accompanying loss of cells in the outer nuclear layer. Furthermore, the number of TUNEL-positive and active caspase-3-immunoreactive cells was increased in the ganglion cell layer. Apoptosis of cells in the retinal ganglion cell layer was further confirmed by the appearance of fragmented nuclei in electron micrographs. Further studies using more reliable quantification of subtypes of neurons in mouse retinas will expand our knowledge of diabetic retinal neuropathy [79]. Taken together these data suggest that retinal degeneration occurs in more than one model of diabetes. Importantly, the increase in apoptosis occurs soon after the onset of experimental diabetes, corresponding to the earliest changes in vascular permeability.

The onset and time-course of apoptosis in the human retina is not as well defined. The number of TUNEL labeled cells was, however, greater in a small number of whole-mounted retinas from human donors. There were 62.2 TUNEL positive cells/0.5 cm$^2$ in a retina from a donor with only 6 years of diabetes and 83.0/0.5 cm$^2$ in another after 30 years of diabetes, compared to 36.4 and 21.6 apoptotic cells/0.5 cm$^2$ in two age-matched non-diabetic donors [71]. Therefore it seems likely that apoptosis of neural cells due to diabetes occurs in humans with a similar frequency and time of onset as in rodents.

A number of other studies have confirmed that diabetes results in apoptosis of a variety of cells in the retina. The reduction in the number of cell bodies in the retinal ganglion and inner nuclear layers of the retinas of rats, after 1 and 4 months of STZ-diabetes, was corroborated by immunohistochemistry for NeuN, a cell-specific marker expressed exclusively in the nuclei of neurons [80]. The total number of inner nuclear layer cell bodies with NeuN immunoreactivity was reduced to approximately 50% of controls after 4, 6 and 12 months of diabetes, accompanied by a 20% reduction in NeuN positive cells in the retinal ganglion cell layer after 1 and 4 months. There was no significant loss of cells in the inner nuclear layer after 1 month of diabetes in this study. Since no reduction in cell number in the retinal ganglion cell layer was evident after 6 months of diabetes the authors suggested that neuronal apoptosis in this region may be limited to shorter durations of diabetes. While this is good evidence that retinal ganglion cells are vulnerable in diabetes it is conceivable that other neurons, such as displaced amacrine cells, would appear positive for NeuN in the retinal ganglion cell layer. However, a loss of retinal ganglion cell bodies is also indicated by a reduction in the number of axons in the optic nerve of rats after 12 weeks of STZ-diabetes [81], and by a clinical study reporting a reduction in the thickness of the retinal nerve fiber layer, using scanning laser polarimetry. In a group of subjects with an average of 15 years of diabetes, the thickness of the nerve fiber layer in the superior polar quadrant of the retina was significantly reduced compared to the control group, indicating a loss of axons in this region and implying an accompanying loss of
retinal ganglion cells [82]. Interestingly, an earlier study of 137 patients with type II diabetes and 144 non-diabetic subjects suggested that nerve-fiber layer defects occurred in 20% of the subjects with no evidence of microaneurysms [83], suggesting that the onset of this defect may occur before, or in parallel with, the earliest vascular changes.

The evidence clearly suggests that diabetes causes apoptosis of the retina, however, there is a degree of disparity in the data concerning the cell types most commonly affected and also the magnitude of cell loss (Table 1 summarizes the results of studies using the TUNEL method to detect apoptosis in retinas from humans and animals with diabetes). This disparity is not uncommon in studies using animal models of diabetes, which vary widely despite the apparent simplicity of modeling diabetes in animals by STZ induction. While strain differences may be one explanation for these variations it is also worth noting that the degree of diabetes obtained by STZ injection can vary widely [84]. It is also the case that several technical considerations can influence the positive number of cells recorded using TUNEL. In our experience a number of experimental variables can affect the outcome of TUNEL quantification, which include: duration and type of fixation; mechanical manipulation of the tissue (poor handling during dissection and mounting the tissue); storage temperature (freezing the tissue should be avoided, and can pose a problem for TUNEL labeling on cryostat sections); terminal transferase enzyme quality (older or incorrectly stored enzyme can also provide false negative results). Therefore, great care should be taken to apply this technique consistently, with appropriate positive and negative control samples included in every experiment.

**Biochemical evidence that diabetes increases apoptosis in the retina**

Because of the subjective nature of quantification using TUNEL labeling, a number of studies have examined other biochemical markers of apoptosis in retinas of diabetic and control rats. BAX immunoreactivity, detected by immunohistochemistry, was increased in 18 postmortem retinas compared to 20 samples from non-diabetic donors, while the pro-survival factor, Bcl-X, was not changed [41]. The immunoreactivity localized to both vascular and neural cells of the inner retina, suggesting a widespread tendency for cells to approach a state of apoptosis. BAX and Bcl-X could not be quantified in lysates of retinal vessels, demonstrating the difficulties in measuring these small chronic degenerative changes, particularly in human tissue where samples are limited in number. In another rat study the amount of BAX associated with mitochondria was also increased after 8 months but not 2 months of STZ-diabetes, compared to controls, suggesting that long durations of diabetes may be required to detect significant mitochondrial dysfunction.
Table 1. Studies reporting TUNEL-positive cells in retinas from diabetic humans and animals.

<table>
<thead>
<tr>
<th>Species/model</th>
<th>Approach</th>
<th>Results</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Human</td>
<td>histological sections</td>
<td>positive cells noted in retinas of subjects with diabetic retinopathy</td>
<td>[69]</td>
</tr>
<tr>
<td>Human</td>
<td>trypsin digest</td>
<td>1.3±1.4 and 13±12 cells in non-diabetic and diabetic groups respectively</td>
<td>[28]</td>
</tr>
<tr>
<td>Human</td>
<td>trypsin digest, 1/6 area of retina</td>
<td>1±1 and 5±4 positive cells in non-diabetic (n=4) and diabetic (n=3) groups respectively</td>
<td>[41]</td>
</tr>
<tr>
<td>Human</td>
<td>whole-mount retinas</td>
<td>58±14.8 and 145±20.8 cells/cm² in non-diabetic (n=2) and diabetic (n=2) groups respectively</td>
<td>[71]</td>
</tr>
<tr>
<td>STZ-rats</td>
<td>histological sections</td>
<td>positive retinal ganglion and Müller cells noted in sections from diabetic rats</td>
<td>[68]</td>
</tr>
<tr>
<td>STZ-rats</td>
<td>histological sections</td>
<td>positive cells noted in outer nuclear layer and other regions</td>
<td>[85]</td>
</tr>
<tr>
<td>STZ-mice, 14 wks diabetes</td>
<td>histological sections</td>
<td>positive cells noted in retinal ganglion cell layer</td>
<td>[78]</td>
</tr>
<tr>
<td>KKA Y mice, 1-3 mth type II diabetes</td>
<td>histological sections</td>
<td>positive cells noted in retinal ganglion cell and inner nuclear layers</td>
<td>[86]</td>
</tr>
<tr>
<td>STZ-rats, 31 wks diabetes</td>
<td>trypsin digest</td>
<td>0.4±0.9 and 9±6 cells in control and diabetic groups respectively</td>
<td>[28]</td>
</tr>
<tr>
<td>Alloxan rats, 6-8 mth diabetes</td>
<td>trypsin digest</td>
<td>0.8±1.3 positive cells in control group, 8.8±6.5 cells in diabetic group</td>
<td>[32]</td>
</tr>
<tr>
<td>STZ-rats, 11 mth diabetes</td>
<td>trypsin digest</td>
<td>2.1±1.4 and 4.1±2.2 positive cells per retina in control and diabetic groups respectively</td>
<td>[33]</td>
</tr>
<tr>
<td>STZ-rats, 1-12 mth diabetes</td>
<td>whole-mount retinas</td>
<td>approx. 20 and 80 positive cells/0.5 cm² in control and diabetic groups respectively</td>
<td>[71]</td>
</tr>
<tr>
<td>STZ-mice, 10 and 24 wks diabetes</td>
<td>whole-mount retinas</td>
<td>approx. 4-12 positive cell in controls, no significant difference in diabetics</td>
<td>[87]</td>
</tr>
<tr>
<td>STZ-rats</td>
<td>whole-mount retinas</td>
<td>approx. 25 and 140 positive cells in control and diabetic group respectively</td>
<td>[87]</td>
</tr>
<tr>
<td>STZ-rats, 1 mth diabetes</td>
<td>whole-mount retinas</td>
<td>4.4±1.7 and 33.3±15.1 positive cells /0.5cm² in control and diabetic groups respectively</td>
<td>[72]</td>
</tr>
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The amount of cytoplasmic cytochrome C, another pro-apoptosis signal associated with mitochondrial dysfunction, was also increased after 8 months of alloxan-diabetes in rat retinas, but not after 2 months [38].

Caspase enzyme activity has also been used as an index of apoptosis. In retinal homogenates from STZ-diabetic rats, caspase -1, -2, -6, -8 and -9 were
more active after 2 months of hyperglycemia, while caspase-3 activity was elevated only after longer durations of STZ-diabetes [88]. Since caspase-3 was increased at a time that correlates with increases in vascular apoptosis it was suggested that this "executioner" enzyme may be more relevant to apoptosis of endothelial cells rather than other cells in the retina. The effects of several dietary antioxidants on caspase-3 activity in alloxan-diabetic rats were examined in another study [37]. Here capase-3 activity was increased only in homogenates of whole retina after 14 months of hyperglycemia. Another study using a similar enzyme activity assay found an increase in caspase-3 enzyme activity in STZ-diabetic rat retinas after 3 months of hyperglycemia. Caspase-3 activity was reversed by the anti-inflammatory drug, minocycline, suggesting that retinal apoptosis may be a consequence of the inflammatory response that appears to play an important role in diabetic retinopathy [89].

Apoptosis has been identified in postmortem human retinas by several studies using TUNEL labeling [28, 69, 71]. Another extensive study used immunohistochemistry for several markers of apoptosis in eyes from five human subjects with between 14 and 20 years of diabetes, compared to eyes from four non-diabetic subjects matched for age and sex [90]. Retinas from diabetic subjects had more cytoplasmic immunoreactivity for caspase-3, Fas, and BAX in the ganglion cell layer. Immunoreactivity for Fas-ligand and Bcl-2 was also detected in glial cells, and ERK immunoreactivity was noted throughout the nuclei of the inner nuclear layer, ganglion cell layer and Müller cell processes. While some of the subjects in this study had proliferative diabetic retinopathy, indicating that their disease had significantly progressed, the results suggest that diabetes causes neurons to express more pro-apoptotic proteins, while glial cells tend to express more anti-apoptotic or cytoprotective markers. Therefore, neurons of the retina, particularly ganglion cells, may be more vulnerable to apoptosis than glial cells, at least in diabetes.

**Identifying the cell types most affected by apoptosis in diabetes**

Several changes in the neural retina are associated with short durations of diabetes in animal models, including increased neural apoptosis and thinning of the inner plexiform and inner nuclear layers [71]. The majority of TUNEL positive cells did not colocalize with the endothelial cell-specific marker, Von Willebrand factor, suggesting that most of the cells undergoing apoptosis were not vascular and therefore more likely to be either neurons or glial cells. In addition, there was a 50% decrease in the number of neurons in the inner nuclear layer after 4 months of STZ-diabetes in rats, suggesting a loss of neurons or Müller cells [80]. The inner retina is made up of several different types of neurons, including horizontal, bipolar, amacrine and ganglion cells. Each has several subtypes with specific roles in visual signal processing, thus
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degeneration or loss of function in any one type of neuron has the potential to cause detectable changes in retinal function. The question of exactly which cells undergo apoptosis, and whether there is a sequential degeneration in which some classes of neurons are lost as a secondary consequence of apoptosis of other cells, remains to be addressed.

Several studies have demonstrated dysfunction or loss of amacrine cells in the retina of diabetic animals. In diabetes, the dopaminergic amacrine cell is most commonly studied. Dopaminergic amacrine cells are essential neurons with many roles in retinal function. Loss of tyrosine hyroxylase activity in a variety of animal models of diabetes strongly suggests that dopaminergic amacrine cells undergo degeneration, or loss of function [91-94]. Other amacrine cells that may undergo apoptosis during diabetes are those that use acetylcholine as their neurotransmitter. In the retinas of diabetic mice, acetylcholinesterase activity, a measure of cholinergic neurotransmission, is decreased [95, 96], suggesting that cholinergic signaling is compromised, or that there is a loss of this subtype of amacrine cells. It is likely that even a limited reduction in the number of cholinergic amacrine cells can compromise vision, because loss of cholinergic neurons or inhibition of cholinergic receptors results in abnormalities in retinal ganglion cell responses [97-99]. Another less common neuron that may be lost during diabetes is the nitric oxide-containing amacrine cell. In STZ-diabetic rats, the number of NADPH diaphorase positive amacrine cells were also decreased [100], but another study measured a paradoxical increase in nitric oxide synthase activity [101]. Some types of amacrine cell also use neuropeptide cotransmitters such as substance P and vasoactive intestinal polypeptide, which may also be significantly reduced in diabetes [102]. Therefore, there is strong evidence for a specific loss of a variety of amacrine cells, which may contribute in a major way to vision loss in diabetes.

Other studies have suggested that apoptosis of photoreceptors also contributes to vision loss in diabetic retinopathy. It has been suggested that a specific loss of function occurs in short wavelength S-cones, which detect blue light, in the human retina [103]. TUNEL-positive cells were found in the inner and outer nuclear layers of postmortem retinas from humans with diabetes. Carbonic anhydrase staining indicated a reduction in the number of S-cones, which may explain the apparent tritanopia, a defect in blue-yellow color discrimination, which can occur in diabetes [13, 104]. An increase in foveal tritanopia in people with diabetes may also be explained by damage to the S-cone pathway [105].

More evidence of photoreceptor apoptosis has been obtained from animal models of diabetes. There was a reduction in the number of cone photoreceptors measured by peanut agglutinin binding, accompanied by abnormalities in the retinal pigment epithelial cells, revealed by electronmicroscopy in retinas of
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STZ-rats after 1 month of diabetes [77]. Extensive TUNEL labeling of the outer nuclear layer of retinas from STZ-diabetic rats after 24 weeks of hyperglycemia has also been reported [85]. Electron microscopy indicated a loss of post-synaptic processes of horizontal cells in the photoreceptor invaginations as little as one week after the onset of diabetes. Similar reduction of the thickness of the outer nuclear layer was also measured in STZ-diabetic mice [78]. It has been suggested that laser photoagulation slows diabetic retinopathy by causing an upregulation of basic fibroblast growth factor in cells at the edge of the laser burn, resulting in protection of the remaining photoreceptors from further apoptosis [106], or that photocoagulation reduces the total energetic needs of the retinal tissue by destroying peripheral photoreceptors [107].

A common approach to quantifying loss of inner retinal function in human and experimental diabetes is the use of the electroretinogram (ERG), which gives a general measure of retinal function in response to a light stimulus [15]. Photoreceptor function was assessed by isolating the photoreceptor responses from the dark-adapted ERG in STZ-diabetic rats with between 4 and 12 weeks of diabetes. The results suggested a photoreceptor pathology, which could be reversed with perintopil, an angiotensin converting enzyme inhibitor [108]. In diabetes, the oscillatory potentials of the ERG, which are likely due to inner retinal neurotransmission [109], have prolonged peak latencies and decreased amplitudes [110, 111], suggesting abnormal inner retinal function. The exact mechanism of these deficits is not known, but the source of oscillatory potentials is thought to be from both amacrine and Muller cells [112]. Thus, the degeneration or loss of one or both of these groups of cells may cause the observed changes to oscillatory potentials. A slight delay in the a-wave response was also noted in another ERG study in STZ-diabetic rats [110], although the sensitivity of this response was not altered and this deficit is not typically noted in other ERG studies of diabetes.

**Potential mechanisms of retinal cell apoptosis in diabetes**

Neuronal apoptosis in the central nervous system is often associated with increased release of extracellular glutamate, which is thought to play a central role in both chronic and acute neurodegeneration of the central nervous system [113, 114]. A number of studies in both rats and humans suggest that the total content of glutamate in the retina and vitreous is elevated by diabetes [60, 115, 116]. Diabetes also reduces the rate at which explant retinas convert $^{14}$C-glutamate into $^{14}$C-glutamine, suggesting that the activity of glutamine synthetase is reduced. The 35% loss in activity of this Müller cell-specific enzyme was also accompanied by a reduction in protein content measured by western blot [115]. The ability of explant retinas to dispose of glutamate by oxidation to alpha-ketoglutarate was also reduced by diabetes. Conversion of
14C-glutamate to 14CO2 through the TCA cycle was reduced to 62% of the control value in retinas from STZ-diabetic rats [117]. Interestingly, the general transaminase inhibitor, aminoxyacetate, blocked the oxidation of glutamate in control retinas but caused no further deficit in retinas from diabetic rats, suggesting that the reduction in the rate of glutamate oxidation is due to impaired transamination of glutamate, which may be a regulatory mechanism for glutamate synthesis and disposal in the retina and brain [118-120]. It has also been suggested that diabetes reduces glutamate transport mechanisms in retinal glia, because dissociated Müller cells from STZ-diabetic rats absorbed 67% less radio-labeled glutamate from the cell-culture media, compared to similarly dissociated cells from control retinas [121]. Taken together these data suggest that diabetes may impair glutamate uptake and metabolism, resulting in a potential accumulation of extracellular glutamate, leading to chronic excitotoxicity.

As well as a potential imbalance in the glutamate-glutamine cycle, current studies suggest that glucose may alter the regulation of AMPA receptor sub-units in retinal neurons, which could lead to a reduction in post-synaptic sensitivity to glutamate [122]. Similar studies have also found changes in glutamate receptors in the brain of STZ-diabetic rats. AMPA receptor binding was reduced in the hippocampus of STZ-diabetic rats, possibly as a consequence of changes in the amount of synaptic glutamate [123]. The content of the NMDA receptor complex was also reduced in the hippocampus of STZ-diabetic rats, accompanied by alterations in long-term potentiation [124]. Other studies of the hippocampus of STZ-diabetic rats also found altered transcription and post-transcriptional modification of the NMDA receptor, as well as increased apoptosis and caspase-3 activity in the hippocampus [125, 126]. Conversely, NMDA and AMPA receptor binding was increased in the spinal cord of ob/ob diabetic mice, which are considered a model of type II diabetes [127]. Changes in glutamate receptor content and binding may be a response to altered glutamate metabolism (or vice versa) and if similar changes occur in the retina this could provide a mechanism for both the increase in apoptosis and the loss of neuronal function.

Another popular hypothesis to explain neural apoptosis is that diabetes reduces the abundance of trophic factors, or the effectiveness of growth factor signaling mechanisms that are essential for survival of neurons in the retina. One of the early studies of apoptosis in STZ-diabetic rats suggested that injection with nerve growth factor protected the retina from apoptosis [68], although in another study nerve growth factor content and its receptor expression were unchanged by diabetes in the retinas of BB/Worster rats, a model of spontaneous diabetes [128]. In another study nerve growth factor reduced the production of lipid peroxides induced by exposure to hydrogen
peroxide in retinal Müller cells [129], suggesting that nerve growth factor may have protective effects on retinal glia as well as neurons.

Another neurotrophic factor that has received attention in studies of apoptosis in the retina is brain derived neurotrophic factor (BDNF). It is well established that BDNF can aid the survival and differentiation of retinal ganglion cells placed in culture [130]. BDNF also acts as a neuroprotective factor for RGC5 cells, an immortalized cell line derived from retinal ganglion cells [131]. There have been suggestions that diabetes depletes the content of BDNF in both the brain and retina [132, 133]. In one study intravitreal replacement of BDNF reduced the loss of dopaminergic amacrine cells, indicated by immunoreactivity for tyrosine hydroxylase, suggesting that this growth factor may be particularly important for amacrine cell survival in the inner retina [133].

While not usually considered a growth factor, it has also been suggested that insulin acts as a survival factor for retinal neurons [134]. There is an abundance of insulin receptors in the inner retina, which respond to physiological concentrations of insulin (10nM) by phosphorylation of Akt [135]. Insulin signaling has been studied in R28 cells, a cell-line isolated from postnatal rat retinas and immortalized by transfection of the viral E1A vector [136]. While R28 cells were derived from a mixed population of retinal cells and have been reported to express glial antigens under certain circumstances [137], they have also been noted to express neuron-specific markers [138]. When apoptosis was induced by serum-deprivation in R28 cells, insulin reduced the amount of apoptosis by activating the PI3 kinase/Akt-survival pathway [139]. The protective ability of insulin to rescue R28 cells from apoptosis was attenuated by 24 hr incubation in 20 mM glucose media, although the glucose alone did not induce apoptosis. Similar but more extreme results were obtained by incubation with glucosamine, suggesting that excess flux through the hexosamine pathway was responsible for the failure in insulin signaling [140]. These data indicate that elevated glucose may reduce the growth factor-stimulated Akt-signaling pathway in the retina in diabetes.

Many studies have focused on the possibility that cellular dysfunction in diabetic retinopathy, and other complications of diabetes, is caused by oxidative stress induced by hyperglycemia [141-144]. Direct measurement of free radicals in tissue is difficult, however, indirect evidence of oxidative stress in the retinas of diabetic animals have been reported. The intracellular antioxidant, glutathione, was depleted after 2 months of STZ-diabetes in rat retinas, but not brain, suggesting that oxidative stress may be more pronounced in the retina [145]. After a similar duration of STZ-diabetes in another study, lipid peroxides measured by thiobarbituric acid reactive substances, were also found to be increased [146]. In one study, caspase-3 activity was measured after 2 and 14 months of diabetes in alloxan-injected rats fed with a complex of
Antioxidants including Trolox, alpha-tocopherol, acetyl cysteine, ascorbic acid, beta-carotene and selenium [37]. The antioxidants reduced caspase-3 activity in the rats after 14 months of diabetes suggesting that an excess of oxidative free radicals may be responsible for retinal apoptosis. Similar antioxidants also reduced apoptosis of pericytes and endothelial cells cultured in 25 mM glucose media. STZ-rats given the antioxidant alpha-lipoic acid for 11 months also had reduced TUNEL labeling and fewer acellular capillaries in the vasculature of trypsin digest retinas, possibly by inhibiting the oxidation of DNA and nitrotyrosine, although the actual number of TUNEL-positive cells was quite small [33].

Antioxidant therapy may also reduce the inflammatory component of diabetic retinopathy. Intravitreal injection of IL-1 in normal rats increased the number of TUNEL positive vascular cells in trypsin digests and also increased 8-hydroxy-2'-deoxyguanosine, an indicator of increased oxidative free radicals [147]. Furthermore, 2 months of STZ-diabetes increased IL-1 activity in rat retinas [148]. In another study antioxidants reduced NF-kappa B activation in alloxan-diabetic rats [149].

One likely source of free radicals is from a malfunction of oxidative metabolism in mitochondria. In a study examining retinal mitochondria there was an increase in release of cytochrome C and a greater association of BAX with mitochondrial membranes, which are both characteristics of apoptosis, in retinas of STZ-diabetic rats after 8 but not 2 months of diabetes [38]. Similar results were also obtained from mitochondria of retinal endothelial cells cultured in 20 mM glucose, and the cytochrome C release could be reversed by inclusion of super oxide dismutase, suggesting that oxidative stress was responsible for the mitochondrial changes that can trigger apoptosis.

Another theory, related to the hypothesis of free radicals in diabetic complications, involves advanced glycosylation end products (AGEs), formed by non-enzymatic interactions between protein and abnormally high concentrations of glucose. Cultured endothelial cells exposed to AGEs have increased apoptosis [150]. Pericytes may be more sensitive to AGEs than other types of cells [151]. AGEs cause downregulation of Bcl-2 in pericytes and this can be reversed by the growth factor pigment epithelium derived factor [152]. As with other sources of free radicals, AGEs have been difficult to measure directly in tissue. The degree of autofluorescence in tissue may be one index of AGE accumulation, and was increased in retinas of STZ-rats [153]. Antibodies have been raised to certain species of AGEs and have been used to measure increased AGEs in retinas of STZ-diabetic rats by dot blot and immunohistochemistry [154].

There are a number of related biochemical theories of diabetic complications involving oxidative stress causing changes in the activity of several enzymes, including glyceraldehyde phosphate dehydrogenase, aldose
reductase, protein kinase C, and AGE formation [144]. It is conceivable that apoptosis could be a direct result of these mechanisms or that there are other causes, such as glutamate excitotoxicity or loss of survival factors. It is also conceivable that apoptosis in diabetic retinopathy is due to a combination of many of these mechanisms.

Summary and conclusion

There is now abundant evidence that apoptosis plays a role in the pathology of diabetic retinopathy. The onset of apoptosis may begin soon after the onset of diabetes. It is also clear that many types of cells are involved, although possibly to differing degrees. Neurons of the inner retina, including amacrine and ganglion cells are lost. Photoreceptor apoptosis may also play a specific role in the loss of vision, while the involvement of bipolar and horizontal cells is less well established. A loss of neurons and photoreceptors in the retina provides a reasonable mechanism for many of the changes in vision function encountered during diabetes, such as loss of contrast sensitivity and reductions in specific components of the ERG. It also seems likely that diabetes increases apoptosis of vascular cells, including endothelia and pericytes, but the functional consequences of apoptosis in vascular cells are less apparent.

The number of cells undergoing apoptosis in the retina at any one time is quite low, giving rise to a chronic degeneration that may only reach physiological significance over several months in animal models and years in humans. However, the chronic nature of the degeneration means that there is a broad opportunity to prevent progress of the disease. Future cytoprotective treatments should be implemented early, to delay the onset of diabetic retinopathy, rather than after the disease has progressed for many years, because healthy retinal function will depend on the survival of a critical number of cells.

We have reached a defining point in diabetic retinopathy research. Many aspects of this complex disease have been identified, including inflammation, vascular permeability, vascular proliferation, glial abnormalities, and apoptosis of multiple cell types. Now we must determine the causal relationships between these different features, as well as the mechanisms that initiate the disease. Future studies of apoptosis and retinal degeneration in diabetes must examine the mechanisms that trigger apoptosis and look to cytoprotective treatments that can be administered as early interventions to prevent the onset of vision loss. A new treatment for the leading cause of vision loss in working-age people may directly target apoptosis or may act through other mechanisms, such as preserving the blood-retinal barrier, but the ultimate goal of any new treatment must be to prevent the neurovascular degeneration and provide healthy vision for all people with diabetes.
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