

SYMPOSIUM REPORT

Retinal ganglion cells in diabetes

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Diabetic retinopathy has long been recognized as a vascular disease that develops in most patients, and it was believed that the visual dysfunction that develops in some diabetics was due to the vascular lesions used to characterize the disease. It is becoming increasingly clear that neuronal cells of the retina also are affected by diabetes, resulting in dysfunction and even degeneration of some neuronal cells. Retinal ganglion cells (RGCs) are the best studied of the retinal neurons with respect to the effect of diabetes. Although investigations are providing new information about RGCs in diabetes, including therapies to inhibit the neurodegeneration, critical information about the function, anatomy and response properties of these cells is yet needed to understand the relationship between RGC changes and visual dysfunction in diabetes.

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Diabetic retinopathy remains a major cause of morbidity in diabetic patients. To date, the retinopathy has been defined based on lesions that are clinically demonstrable, and all of those have been vascular in nature. Thus, lesions indicative of the early stages of retinopathy include capillary degeneration (which, if it later becomes extensive, can contribute to retinal ischaemia and subsequent neovascularization) as well as retinal oedema, cotton wool spots, haemorrhage, and hard exudates. Available clinical evidence strongly suggests that the late, clinically meaningful stages of the retinopathy are a direct consequence of the earlier changes. Development and progress of retinopathy can be slowed by intensive insulin therapy if administered from the onset of diabetes (Engerman & Kern, 1987; Diabetes Control and Complications Trial Research Group, 1993), but it remains very difficult for many patients to achieve and maintain intensive glycaemic control. More recent clinical studies have also demonstrated that blood pressure medications significantly slow the progression to the late, proliferative stages of diabetic retinopathy (Chaturvedi *et al.* 1998; UK

Prospective Diabetes Study Group, 1998*a,b*; Chaturvedi, 2000).

The clinically demonstrable changes to the retinal vasculature in diabetes have led to the general assumption that the retinopathy is solely a microvascular disease. Nevertheless, diabetes can also damage non-vascular cells of the retina, resulting in alterations in function (Shirao & Kawasaki, 1998; Li *et al.* 2002; Hancock & Kraft, 2004; Barile *et al.* 2005; Phipps *et al.* 2006; Kern *et al.* 2007) and in loss of ganglion cells, horizontal cells, amacrine cells and photoreceptors (Sima *et al.* 1992; Kamijo *et al.* 1993; Hammes *et al.* 1995; Barber *et al.* 1998; Lieth *et al.* 2000; Zeng *et al.* 2000; Aizu *et al.* 2002; Asnaghi *et al.* 2003; Park *et al.* 2003; Kusner *et al.* 2004; Martin *et al.* 2004; Ning *et al.* 2004; Seki *et al.* 2004; Gastinger *et al.* 2006). Thus, diabetic retinopathy can include changes to the neural retina. This review will focus on the effects of diabetes on retinal ganglion cells.

Diabetes increases cell death of retinal ganglion cells: humans

Several studies of histological material have demonstrated that retinal ganglion cells seem to be lost in diabetic patients (Table 1). Moreover, *in vivo* use of scanning laser polarimetry and other techniques found a thinning of the thickness of the nerve fibre layer in diabetes, further consistent with loss of RGCs and their axons in diabetes. In many of these studies, the type of diabetes was not

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Table 1. Evidence suggesting diabetes-induced degeneration of RGCs in humans

| Method | Observation | Reference |
|---|--|--|
| Histology of autopsy samples | Atrophy of RGC, degeneration of NFL | Wolter 1961; Bloodworth, 1962; Kerrigan <i>et al.</i> 1997 |
| Immunohistochemistry of autopsy samples | Apoptosis of RGC, overexpression of Bax, and activated caspase-9 and -3 | Barber <i>et al.</i> 1998; Abu-El-Asrar <i>et al.</i> 2004; Abu-El-Asrar <i>et al.</i> 2007; Oshitari <i>et al.</i> 2008 |
| NFL defects detected by red-free photography | Detectable in 20% of diabetics without microaneurysms, and 57% with microaneurysms | Chihara <i>et al.</i> 1993 |
| NFL 'thickness' from scanning laser polarimetry | Decreased in diabetic patients, and related to severity of retinopathy | Chihara & Zhang, 1998; Ozdek <i>et al.</i> 2002; Takahashi <i>et al.</i> 2006 |
| NFL 'thickness' from scanning laser polarimetry | Decreased in superior retina of diabetic patients | Lopes de Faria <i>et al.</i> 2002 |

Table 2. RGC death or atrophy in diabetic rats

| Model | Duration | Method | Reference |
|-----------|-------------|---|-------------------------------|
| STZ rats | 8–12 weeks | % of optic nerve axons | Scott <i>et al.</i> 1986 |
| BB/W rats | 12 months | Decrease in diameter but not density of optic nerve axons | Sima <i>et al.</i> 1992 |
| STZ rats | 7.5 months | TUNEL, RGC count | Barber <i>et al.</i> 1998 |
| STZ rats | 1–12 months | NeuN immunohistochemistry | Zeng <i>et al.</i> 2000 |
| STZ rats | 4 weeks | TUNEL | El-Remessy <i>et al.</i> 2006 |
| STZ rats | 12 weeks | TUNEL | Seigel <i>et al.</i> 2006 |
| STZ rats | 3 months | Thy-1 immunostain | Qin <i>et al.</i> 2006 |
| STZ rats | 4–5 weeks | Retrograde dye | Kusari <i>et al.</i> 2007 |
| STZ rats | 9 months | Count RGC | Zheng <i>et al.</i> 2007b |

stipulated, but this omission seems not to be critical, since clinical evidence to date suggests that the retinopathy that develops in type 1 diabetes is indistinguishable from that which develops in type 2 diabetes.

Immunohistochemical studies of cross-sections of human retinas demonstrated an increase in expression of Bax, caspase-3 and caspase-9 in RGCs from diabetic patients (Oshitari *et al.* 2008), thus suggesting that at least some retinal ganglion cells might die via apoptosis. In addition, RGCs and occasional cells in the inner nuclear layer showed increased immunostaining for Bad, cytochrome c, and AIF in retinas from diabetic patients. Expression of Cox-2, Akt, and Mcl-1 was not altered in the diabetic retinas (Abu El-Asrar *et al.* 2007). Together these studies show that RGCs undergo apoptosis in humans with diabetes, leading to a reduction in the thickness of the nerve fibre layer.

Experimental diabetes causes degeneration of retinal ganglion cells in rats

The majority of studies of retinal ganglion cells in rats have utilized a chemical (streptozotocin; STZ) that is toxic to pancreatic β cells, thus causing insulin-deficient diabetes that resembles type 1 diabetes. The insulin deficiency that results after STZ in rats can be profound, so small

doses of exogenous insulin are often administered to avoid diabetes-induced weight loss, dysmetabolism, severe polyuria and hyperglycaemia. The BB/W rat likewise is a model of type 1 diabetes, but develops diabetes spontaneously. Models of type 2 diabetes have not yet been used to examine changes in RGC physiology and survival.

RGC loss in diabetic rats. There is general agreement that all rat strains reported to date have shown RGC loss or damage in diabetes (Table 2). Consistent with a possible role of apoptosis in the death of retinal neurons, numerous initiator and effector caspases have been found to become activated in retinas of diabetic animals (Kowluru & Koppolu, 2002; Mohr *et al.* 2002), and neurons become TUNEL-positive or caspase 3-positive in retinas of diabetic rats (Barber *et al.* 1998; Lieth *et al.* 2000; Barber *et al.* 2005; Gastingier *et al.* 2006). In both neuronal and capillary cells, however, the number of cells dying at any given time is very small, perhaps contributing to the slow onset and progression of diabetic retinopathy.

Further circumstantial evidence of retinal ganglion cell loss or alteration in diabetic rats comes from histological studies of retinal thickness. There was a 22% decrease in the thickness of the inner plexiform layer in rats after 7.5 months of STZ-diabetes, suggesting a cumulative loss of neural dendrites and synapses in

the inner retina (Barber *et al.* 1998). Studies by other investigators have reported similar losses of thickness in the plexiform layers due to experimental diabetes. In retinas of Sprague–Dawley rats there was a 10% reduction in the thickness of the inner plexiform layer reported after only 1 month of STZ-diabetes, while the reduction in the inner plexiform layer of STZ-diabetic Brown–Norway rats was nearly 16% (Aizu *et al.* 2002). While the rate of progression of degeneration appears to vary in different animal models, the data suggest that diabetes causes a progressive loss of the neuronal structures in the inner retina.

Other studies of the nerve fibre layer and optic nerve provide further evidence of RGC loss. The number or density of axons in the rat optic nerve was reduced by STZ-diabetes in some (Scott *et al.* 1986), but not all (Sima *et al.* 1992; Kamijo *et al.* 1993), rodent studies.

Effect of experimental diabetes on retinal ganglion cells in mice

The effect of diabetes on retinal ganglion cells has been studied using models in which diabetes was induced experimentally using streptozotocin to disrupt pancreatic β cells, in mice that spontaneously develop a type 1-like diabetes (Ins2^{Akita}), and in the spontaneously diabetic KKAY strain (a model of Type 2 diabetes).

The spontaneous development of diabetes resulted in loss of cells in the ganglion cell layer in Ins2^{Akita} mice (Barber *et al.* 2005). After 22 weeks of hyperglycaemia, there was a 23.4% reduction in the number of cell bodies in the retinal ganglion cell layer. This was accompanied by a significant reduction in the thickness of the inner plexiform layer in these animals. By crossing Ins2^{Akita} with mice that express fluorescent proteins under the regulation of the Thy1 promoter, it was possible to quantify the specific loss of retinal ganglion cells (Gastinger *et al.* 2008). Cyan fluorescent protein (CFP) was expressed in the cell bodies of about 50% of all RGCs, and quantification of the number of fluorescent cells surviving after 3 months of diabetes revealed 16% depletion from the peripheral retina, but no significant loss in the central region. There was a 27% decrease in the thickness of the inner plexiform layer in Ins2^{Akita} mice after 5.5 months of STZ-diabetes, suggesting a cumulative loss of neural dendrites and synapses (Barber *et al.* 2005).

Reductions in thickness of the inner and outer retina have been detected also in C57Bl/6 with diabetes induced using STZ (Martin *et al.* 2004; Zheng *et al.* 2007a). Surprisingly, however, there is controversy with regard to whether or not diabetes causes RGC death in this mouse strain. One group of investigators reported that STZ-diabetes in this strain rapidly resulted in extensive loss of RGCs, resulting in 20–25% fewer cells in the ganglion cell layer compared with age-matched control

mice after only 14 weeks of diabetes (Martin *et al.* 2004), whereas other investigators using this same strain found no evidence of RGC degeneration even after 1 year of diabetes, based on counts of the number of cell bodies in the RGC layer of retinal cross-sections, retrograde labelling of retinal ganglion cells with fluorescent dye, and TUNEL staining of retinal cross-sections (Asnaghi *et al.* 2003; Feit-Leichman *et al.* 2005). A transient increase in TUNEL labelling and caspase-3 activity has been detected in retinas of C57Bl/6 mice shortly after injection of STZ (Feit-Leichman *et al.* 2005), but this did not result in increased TUNEL staining or loss of RGCs after a longer durations of diabetes. This discrepancy with respect to whether or not RGCs die in diabetic C57Bl/6 mice remains difficult to interpret.

The TUNEL technique in whole retinas also demonstrated that apoptosis of RGCs occurs in KKAY mice (Ning *et al.* 2004). While there are some discrepancies in the prevalence of RGC loss in different mouse models of diabetes, some of this may be due to the differential reaction of mice to STZ, which tend to require higher doses and repeated administration to induce hyperglycaemia that is comparable to the rat models.

Specific indications of retinal ganglion cell pathology

More recent data show that retinal ganglion cells in the Ins2^{Akita} mouse retina develop structural alterations of RGCs within 3 months of diabetes. These features include swellings on axons, often associated with constriction close to the cell body (Fig. 1).

Swellings similar to those in RGCs were noted on histamine immunoreactive fibres in the STZ-diabetic rat retina (Gastinger *et al.* 2001). In contrast, yellow fluorescent protein (expressed under the Thy1 promoter to restrict its expression to RGCs) was expressed throughout the RGC cell body and dendrites of a smaller number of RGCs, enabling quantitative analysis of the morphology of these cells (Gastinger *et al.* 2008). In diabetes, there was structural remodelling of dendrites, including an increase in the total length, density, and number of terminals. Interestingly, these changes were limited to the large ON-retinal ganglion cells and did not occur in any class of OFF-ganglion cell. Similar changes in morphology have been observed in human retinas and STZ-rats (Qin *et al.* 2006; Meyer-Rusenberg *et al.* 2007). These data suggest that the morphology of a subset of RGCs is altered by diabetes in such a way that could alter the functional output of certain subtypes of RGCs.

Diabetes has been reported to impair axonal retrograde transport in large- and medium-sized RGCs in type 1 diabetic rats, but not type 2 diabetic rats (Zhang *et al.* 1998, 2000). The defect in axoplasmic flow in RGC from type 1 diabetic rats was normalized using an aldose reductase inhibitor (Ino-Ue *et al.* 2000).

Loss of retinal function in diabetes

The onset of vision loss is insidious in diabetes, commonly beginning with a reduction in night vision or the ability to see details in low light conditions (Bailey & Sparrow, 2001). While clinical diagnosis of diabetic retinopathy requires detection of vascular pathology, the disease also includes deficits in the electroretinogram, and other measures of function such as contrast sensitivity (Della Sala *et al.* 1985; Sokol *et al.* 1985; Tzekov & Arden, 1999), suggesting that acquisition or processing of the visual signal is impaired (Lopes de Faria *et al.* 2001). Such functional changes can occur before the gross vascular defects become detectable clinically (Simonsen, 1980; Juen & Kieselbach, 1990). The oscillatory potentials of the electroretinogram, which are likely to be due to inner retinal neurotransmission (Dong *et al.* 2004), have prolonged peak latencies and/or decreased amplitudes in diabetic rats (Sakai *et al.* 1995; Hancock & Kraft, 2004; Phipps *et al.* 2006; Ramsey *et al.* 2006; Kern *et al.* 2007; Layton *et al.* 2007; Shinoda *et al.* 2007), suggesting abnormal inner retinal function. The origin of the electroretinogram anomalies is not known, but the source of oscillatory potentials may be from synaptic activity between amacrine neurons and bipolar or retinal ganglion cells (Wachtmeister, 1998). These deficits could be explained by degeneration in synaptic neuro-

transmission, or a combined loss of amacrine neurons and RGCs.

Apoptotic loss of RGCs combined with morphological changes in the surviving RGCs may account for some of the functional deficits in diabetes. Nevertheless, diabetes-induced deficits in RGC function might occur before morphologic changes. At present, there have been no detailed studies of RGC function in diabetes.

Experimental therapies resulting in preservation of retinal neurons in diabetes

There is still no consensus on the best pharmacological target for diabetic retinopathy, but apoptosis clearly has been shown to participate in the death of RGCs (Kerrigan *et al.* 1997; Barber *et al.* 1998, 2005; Abu-El-Asrar *et al.* 2004, 2007; Martin *et al.* 2004; Feit-Leichman *et al.* 2005; El-Remessy *et al.* 2006; Ali *et al.* 2008; Oshitari *et al.* 2008).

Metabolic pathways that cause this apoptotic response are largely unknown. Common theories suggest that inflammation, oxidative stress or exposure to advanced glycation end products might contribute to retinal pathologies, including RGC apoptosis. Several experimental therapies have been found to inhibit RGC loss and some other neural changes in animal models

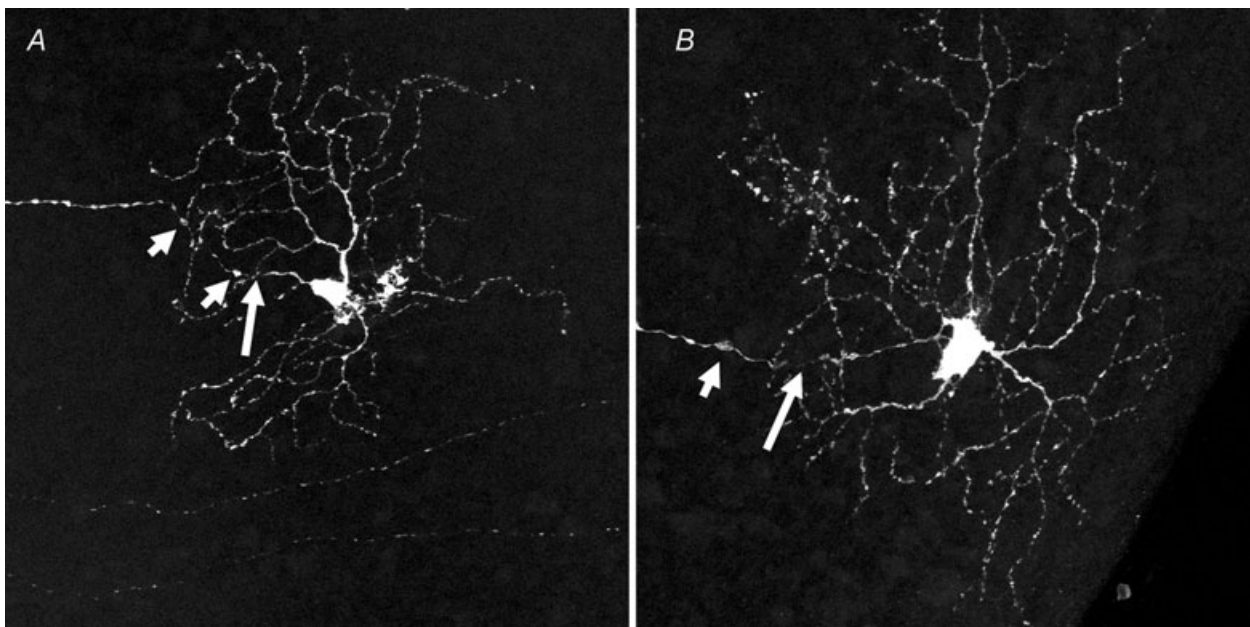


Figure 1. Retinal ganglion cell abnormalities in retinas from diabetic *Ins2^{Akita}* mice

Ins2^{Akita} and Thy1-YFP mice were crossbred to produce spontaneously diabetic animals with endogenous expression of the yellow fluorescent protein in a subset of RGCs. Cells were imaged by confocal microscopy (Leica TCS SP2 AOBS) and rendered as maximum projections from z-stacks that included the axon and entire dendritic field. Abnormal features were noted in the ganglion cells of retinas from mice that were diabetic for three months. The abnormalities included axonal swellings (short arrows) and associated constriction (long arrows), as well as enlarged cell bodies and increased dendritic branches and terminals. A, medium ON-RGC; B, large ON-RGC (Gastinger *et al.* 2008).

of diabetes. Tests with anti-inflammatory drugs such as minocycline and the non-steroidal cyclooxygenase inhibitor nepafenac have yielded positive results (Kradly *et al.* 2005; Kern *et al.* 2007; Vincent & Mohr, 2007). Several salicylates, including some that have little effect on cyclooxygenase, also inhibited the diabetes-induced degeneration of RGCs by a mechanism that seems to involve inhibiting the activation of the proinflammatory NF- κ B (Zheng *et al.* 2007b). Another potential cause of RGC loss is excitotoxicity due to excessive synaptic glutamate activity. There is evidence that diabetes elevates the total amount of glutamate in humans and rats (Ambati *et al.* 1997; Lieth *et al.* 1998; Kowluru *et al.* 2001), while glutamate uptake and processing is also altered (Lieth *et al.* 1998; Li & Puro, 2002; Puro, 2002). The glutamate NMDA receptor antagonist, memantine, is regarded as a neuroprotective drug, and has been shown to reduce the amount of RGC loss in diabetes (Kusari *et al.* 2007). Cannabidiol has been suggested as a neuroprotective therapy for RGC loss in glaucoma (El-Remessy *et al.* 2003), and has also been shown to prevent neural cell death and vascular permeability after a short period of diabetes (El-Remessy *et al.* 2006). In addition, nerve growth factor (Hammes *et al.* 1995), IGF-1 (Seigel *et al.* 2006), aldose reductase inhibitors (Asnaghi *et al.* 2003; Cheung *et al.* 2005), erythropoietin (Zhang *et al.* 2008), and the peroxynitrite decomposition catalyst, FeTTPS (El-Remessy *et al.* 2005), all have been found to inhibit RGC degeneration in diabetes. Aminoguanidine and aldose reductase inhibitors have been found to inhibit atrophy of optic nerve axons (i.e. from the RGCs) in diabetes (Ino-ue *et al.* 1998a,b). Currently these studies are too few to clearly indicate the biochemical mechanism(s) leading to RGC death in diabetes, but do provide important information to initiate further work.

It is important to recognize that most of these studies have focused on preventing RGC degeneration, but effects of these and other therapies on dendritic field morphology and other features important for RGC function have not been assessed. Further studies using neuroprotective drugs should broaden their scope beyond neuronal death to include ganglion cell morphology and function, as well as effects on vascular permeability and structure. Such studies will determine if there is a causal relationship between the vascular and neural changes in diabetic retinopathy.

Conclusions

There is general agreement (with the exception of C57Bl/6 mice) that some RGCs die in diabetes, and some show alterations in structure. The molecular mechanism by which the cells die or develop other structural abnormalities is not yet clear, but inflammation, excitotoxicity, and oxidative/nitrative stress might be

implicated based on the limited number of studies done to date. Importantly, the focus pertaining to RGSs in diabetes should not be restricted to degeneration. Information about diabetes-induced dysfunction, anatomy and response properties is expected to provide considerable insight into the visual dysfunction that develops in some diabetic patients. To accomplish this, more experts and studies on retinal ganglion cell structure, function and physiology are needed to investigate the response of the retina to diabetes. Ultimately, these studies are likely to shed light on the potential role of altered RGC function and survival in diabetes-induced vision loss.

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