



Alistair Barber

# Dual mechanisms may contribute to the pathology of diabetic retinopathy

Gearoid Tuohy PhD  
in Vienna

DEATH and remodelling of retinal ganglion cells may explain a number of early anomalies in visual function brought about by diabetes, suggests new research presented at the Winter EURETINA conference.

Alistair Barber PhD, associate professor of ophthalmology at Penn State College of Medicine, reported many years of work by the Penn State Retina Research Group into neuronal damage associated with diabetic retinopathy (DR). While the disorder has clear vascular and neuronal abnormalities assumed to be intimately connected, Dr Barber told delegates that separate vascular and neuronal mechanisms may be occurring within the retina.

DR affects more than 90 per cent of people with diabetes and, despite significant advances in clinical management, it remains a leading cause of new cases of adult blindness worldwide. It has been well established that the best predictor of DR is the duration of diabetes – for Type 1 diabetes the risk is low over the first five years however, the risk climbs to 27 per cent for those diagnosed for 5-10 years and 71-90 per cent for those with diabetes for 10 years or longer. The incidence rises to 95 per cent for those diagnosed with diabetes for 20-30 years.

Optimal management of DR is achieved through a combination of glucose control, laser therapy and vitrectomy. Regular fundus examination, in addition to adherence to the ETDRS recommendations, greatly reduces vision loss, however the vast majority of lost vision arises from delays in seeking medical attention. Consequently there is significant interest in understanding the complex pathology of DR and the mechanics of molecular pathology in the early stages.

Dr Barber and his research team have been investigating the cell biology of DR attempting to obtain an improved knowledge of the molecular events that might ultimately open diagnostic or therapeutic opportunities. When the Penn State University group initiated their studies into DR one of the big questions posed by the research team was what happens to the neuronal cells? Although there was some data in existence, for example a number of researchers had recognised apoptotic neurons in the retinas of diabetic animals, there was relatively little information on what was occurring at the cellular level.

Textbook schematics of the neural retina illustrate a complex tissue. Photoreceptor cells synapse or connect with bipolar cells and horizontal cells, bipolar cells synapse with retinal ganglion cells (RGCs) which in turn lead to the brain while amacrine

cells are thought to regulate many of these connections. This delicate intricacy illustrates the experimental challenge of teasing out which cells are doing what.

In initial studies Dr Barber's team looked at cross sections of retina from rats with diabetes and over a period of eight months made detailed morphological measurements. Analysis of such measurements showed a clear reduction in the thickness of some of the retinal layers over a period of streptozotocin (STZ) induced diabetes, a well established model for mimicking diabetes. Of all the retinal layers, the inner plexiform layer (IPL) of such animals appeared to be changed the most with almost a 22 per cent reduction in the thickness. Interestingly, the IPL is mostly comprised of synapses, so the loss of thickness hinted at a reduction in neuronal function. The inner nuclear layer (INL) was also reduced in thickness, however there was no observation of thinning seen in the outer layers.

Such histological data showed that something was lost from the retina but it was unclear what specific cells were being removed by the pathology. Measuring apoptosis across the whole retina and counting TUNEL (TdT-mediated dUTP nick end labelling) positive cells revealed that there was significant increases of apoptosis in diabetic animals across all time points studied, which suggested there was something happening quite early on in the retinas of these diabetic animals. Identifying which cells were dying has been a difficult question to answer, explained Dr Barber. Some of the cells appear to be vascular in origin while others are clearly inter-vascular. Detecting active forms of caspase 3, a key molecular player in apoptotic cell death, showed that apoptotic cells were found some distance from the vasculature, implying that these cells were more likely to be neural than vascular.

## Transgenic detective work

To understand more of the cellular biology, Dr Barber and colleagues carried out some transgenic crosses (Ins2Akita/+ X Thy1-CFP or Thy1-YFP [reporter genes]) which allowed researchers to visually track the impact of diabetes on retinal ganglion cells. The "Akita" mice are spontaneously diabetic carrying a point mutation on one of the insulin genes and, while born normal, within four or five weeks of birth they develop diabetes, which persists for the rest of their lives. The Thy 1 promoter is specifically expressed within the retinal ganglion cells and by tagging on -CFP (cyan fluorescent protein) and -YFP (yellow fluorescent protein) markers one can observe and count the number of surviving retinal ganglion cells in the retina.

CFP is expressed in the cell bodies of the RGCs while YFP appears to be expressed throughout the entire neuronal structure. The use of both allows for the detection of morphological events in distinct parts of the cell and crossing the animals then allowed the researchers to track the fate of these cells in a diabetic retina.

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Quantifying the RGCs in eight different regions of the retina (four central, four peripherally) the team found that there was a significant loss of RGCs in the peripheral region but not in the central region. The YFP mice were especially interesting (YFP is expressed throughout the entire structure of the RGC cell) as they showed that swellings occurred along the axons of these cells. Approximately 30 per cent showed swellings that appeared to be associated with narrowing and often the cell bodies appeared to be larger, similar to Wallerian degeneration observed when axons are severed. Dr Barber hypothesised that such swellings might be due to a "train wreck scenario" in which transport of proteins along the axon is blocked, leading to their accumulation, but why this might occur in DR remains unclear. Certainly the morphological data suggested that there might be transport issues in the axons and such interruption may be linked to apoptotic triggers.

Another set of observations included the dendrite structures, axon lengths, branch points and terminals. In diabetic animals there were more terminals, branch points and lengths in the ON cells but not the OFF cells suggesting something specific to functional activity. Such observations gave rise to further studies built around the synapses. As previously established, the inner plexiform layer (IPL) was significantly thinned in the diabetic models and as the IPL is comprised of many synapses it was logical to take a look at pre-synaptic markers such as synapsin I and others. Many such markers appeared to be depleted in the diabetic model and depletion would suggest there

may be some functional consequence for neurotransmission in this cell layer, which might explain some of the changes widely observed in electrophysiology recordings both in humans and in animals.

To drill a little deeper the research team isolated synaptic terminals or "synaptosomes", using a standard technique well established in experimental neurophysiology. Pinching off the synaptosome allows for measurement of the levels of a number of synaptic proteins inside – synaptophysin, synapsin I, VAMP2 and SNAP25 – all involved in different functions of regulating neurotransmission vesicle movement. Remarkably, all such measurements showed significant depletion of synaptic proteins in rat retinas, even after one month of diabetes.

According to Dr Barber, such observations might explain some of the functional effects seen in neuronal cells in the diabetic retina. Synapsin I is phosphorylated and when the synaptosomes are isolated and stimulated by depolarisation with KCl (a classic experiment in neuroscience) phosphorylation of synapsin I occurs within about a minute of depolarisation. This is exactly what is observed in synaptosomes from control animals, however in animals that have been diabetic for only one month the KCl stimulation does not give rise to the same amount of phosphorylation of synapsin I, indicating that there may be some manner of loss of functional activity caused by diabetes.

It is difficult to envision a model in which vascular events influence a reduction in phosphorylation of synapsin. Therefore, it may be time to consider distinct neuronal and vascular mechanisms at play. Identifying such mechanisms represents the first step in devising some strategy to modify the decrease in phosphorylation that might ultimately benefit neuronal function in the diabetic retina.

Dr Barber and his team have built a body of evidence showing that diabetes increases the rate of apoptosis in the retina, that some of the dying cells are neurons; that there is a loss of synapsin I phosphorylation and that this may explain some of the loss in function seen in the early stages of DR.

Dr Barber suggested that it may be time to consider a more unusual possibility, which is that early on in the course of DR there may be separate mechanisms going on in the retinal tissue – vascular events and neuronal events with distinct molecular mechanisms, however difficult it might be to imagine given the general belief that vascular and neuronal pathologies are intimately connected.

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