Realtime Imaging of Retinal Ganglion Cell Apoptosis

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Abstract
Retinal ganglion cell apoptosis has long been highlighted as an important early event in glaucoma. Recent work from our group has shown that it is possible to visualise its occurrence in vivo using detection of apoptosing retinal cells (DARC), a recently devised non-invasive realtime imaging technique using fluorescently labelled annexin V and ophthalmoscopy. To date, DARC has been used only experimentally, but phase I clinical trials are due to start shortly in glaucoma patients. Extrapolation of these initial studies suggests that DARC may provide a new and meaningful clinical end-point in glaucoma, enabling early identification before the onset of irreversible visual loss as well as quantitative tracking of cellular degeneration and response to treatment.

Keywords
Retinal ganglion cell, apoptosis, imaging, glioma, detection of apoptosing retinal cells (DARC)

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The retinal ganglion cell (RGC) is the key cell implicated in the development of blindness in glaucoma.1–4 However, standard clinical tests are believed to identify visual field defects when up to as much as 40% of RGCs are lost, resulting in a potential 10-year delay in glaucoma diagnosis.4–6 Utilising the unique optical properties of the eye, the newly developed detection of apoptosing retinal cells (DARC) technology enables direct visualisation of nerve cells dying through apoptosis, identified by fluorescent labelled annexin V. Our laboratory has assessed DARC in different retinal neuro-degenerative experimental models4,25,26 and highlighted its potential in early diagnosis – in the previously regarded “subclinical” stages of glaucoma. These studies have also demonstrated the use of DARC in assessing neuro-protective strategies.3,9,10,11

Principles of DARC
The process of apoptosis has been identified as the major contributory process to RGC loss in glaucoma.1–4 Annexin V has been used for many years to identify apoptosis in vitro, based on its ability to bind to phosphatidylserine (PS), which becomes externalised in the outer leaflet of cells undergoing the earliest stages of apoptosis.12 More recently, it has been used in vivo, particularly when tagged clinically with technetium-99m (99mTc), with applications in acute myocardial infarction and cardiac allograft rejection, ischaemic brain injury, hepatitis and lung, breast and haematological cancers.13–21 Instead of using 99mTc, our laboratory recently showed that by tagging annexin V with a fluorescent marker, it was possible, using high-resolution imaging, to visualise and track RGC apoptosis.22 This was originally performed using a confocal laser scanning ophthalmoscope (cLSO), with an argon laser of 488nm necessary to excite the administered annexin V-bound fluorophore, and a photodetector system with a 521nm cut-off filter to detect the fluorescent emitted light.4,22 We subsequently used other fluorophores, but the principle of DARC remains the same.22,23

Until now, DARC has only been tested on experimental models.4,9,10,12,23 For imaging, animals are anaesthetised, their pupils are dilated and they are positioned in front of a cLSO. Retinal images are captured using a method we have previously described,24 from which the total number of apoptosing RGCs for each time-point in vivo is calculated, and an average density count per mm² generated (see Figure 1).22 This count may be used to assess disease activity in each eye, along with the response to treatment.4,9,10

Retinal Ganglion Cell (RGC) Apoptosis and RGC Loss in Glaucoma
RGC apoptosis has been identified in clinical and experimental specimen eyes. However, until the development of DARC, evidence for apoptotic RGC death had been restricted to histological and post mortem analysis.1–3,5,6 Nevertheless, the process of RGC apoptosis had been highlighted as one of the earliest hallmarks of the glaucomatous process.6

A study by Quigley et al. in experimentally induced glaucoma in monkeys showed that 4–13% of RGCs were undergoing apoptosis in early disease.1 However, there was at least a 10-fold difference between light microscopy methods compared with terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) analysis.7 Post mortem analysis of specimen eyes from patients with
glaucoma has confirmed the occurrence of RGC apoptosis, although accurate percentage counts are not available.

Several models of ocular hypertension (OHT) have been developed in the rat, of which the technique first described by Morrison et al., and used by the current authors, has become the most popular. The development of RGC loss in this model has been well-documented, with peak RGC loss of around 30–40% occurring at one month after intraocular pressure (IOP) elevation. Within this model, RGC apoptosis occurs predominantly in the early phase of RGC loss in rat OHT, possibly as a pressure-related response. Our studies with DARC in vivo, validated histologically, showed RGC apoptosis rates of 1, 15, 13, 7, and 2% of total RGCs, with RGC losses of 17, 22, 36, 45, and 60% of the original population at two, three, four, eight, and 16 weeks, respectively. This was in comparison with an optic nerve transection model, where RGC apoptosis levels were recorded as 0.3, 1, 8, and 3% of total RGCs, with RGC losses of 0, 3, 40, and 76% at zero, three, seven, and 12 days, respectively.

In estimating the levels of RGC loss, Zeyen was the first to discuss a normal ageing rate of approximately 0.4% loss per year, compared with 4% per year due to glaucoma. In the same paper, he computed that since visual field defects were only detected in glaucoma after a loss of ~40% of RGCs, standard perimetry equated to an approximate 10-year delay in diagnosis. This finding is now supported by other studies. In this extrapolation, we assumed a sudden onset to a hypothetical clinical situation by converting rat years into human years. In this extrapolation, we assumed a sudden onset and development of the glaucomatous disease process in a 50-year-old patient. The rate of RGC loss in such a patient is predicted to change from 0.4% (normal age-related loss) to 4% per year (glaucomatous loss, ) as previously described by Zeyen. RGC numbers have been calculated in Table 1 using these rates, but also taking into account the extrapolated profile of RGC apoptosis shown in Figure 2. From these, levels of RGC apoptosis per year and per day have been calculated.

![Image](Realtime Imaging of Retinal Ganglion Cell Apoptosis)

**Extrapolating DARC to the Patient**

Using the rat model of experimental glaucoma, described above, we have developed an accurate profile of RGC apoptosis following surgical elevation of IOP (see Figure 2). We applied this same profile to a hypothetical clinical situation by converting rat years into human years. In this extrapolation, we assumed a sudden onset and development of the glaucomatous disease process in a 50-year-old patient. The rate of RGC loss in such a patient is predicted to change from 0.4% (normal age-related loss) to 4% per year (glaucomatous loss, ) as previously described by Zeyen. RGC numbers have been calculated in Table 1 using these rates, but also taking into account the extrapolated profile of RGC apoptosis shown in Figure 2. From these, levels of RGC apoptosis per year and per day have been calculated.

![Image](DARC Image of the Retina of Rat Treated with Staurosporine Two Hours Previously)

Figure 3 displays the data in Table 1 graphically. It appears that the daily count of apoptosing RGCs (the DARC count) is much greater than that in an age-matched normal eye—ranging from 50 to 400 cells per day within the first 10 years of disease. Interestingly, this 10-year period coincides exactly with the time-lag currently estimated as the delay in visual field perimeter detecting abnormalities, suggesting DARC may have a role in the detection and diagnosis of early glaucoma.

**Current Clinical End-points in Glaucoma**

At a meeting organised by the US National Eye Institute (NEI)/US Food and Drug Administration (FDA) (13–14 March 2008, Glaucoma Clinical Drug Trial Design and End-points Symposium, Bethesda, US), a clear and unmet need in glaucoma for methods to detect this disease early, before the onset of permanent vision loss, was identified. This has been further highlighted by the recent announcement of discouraging results of the first neuroprotective phase III clinical trial in glaucoma, by Allergan Inc.
The rate of retinal ganglion cell (RGC) loss in glaucoma is predicted to change from 0.4% (normal age-related loss) to 4% per year (glaucomatous loss), as previously described by Zeyen. RGC loss was not. Although the full results have not yet been published, poor end-points may have been a contributory factor — IOP could not be used, so visual fields and optic disc changes were utilised and may have accounted for the long period of follow-up (>5 years) necessary for this trial.

Our group has used DARC to test of the efficacy of neuroprotective treatments in several models of glaucoma.6,32,33,44 In fact, potentially the most immediate benefit of DARC will be in its application to directly monitor the effects of therapy in glaucoma. Glutamate modulation is proposed as a possible target. 

**Into the Future with DARC**

We believe that DARC should provide a snapshot of the number of apoptosing RGC at any one time in patients. As such, it is hoped the DARC count (see Figure 3) will provide a new end-point in glaucoma. However, only the planned large population-based clinical studies will establish the DARC count in relation to glaucoma and the normal ageing process in order to validate the estimates above. It will also permit the investigation of whether a specific pattern of apoptosis occurs, as we postulate it will be along the pathway of retinal nerve fibres, with an increased probability of detecting focal areas of increased DARC activity in the papillo-macular bundle.

As DARC enables direct observation of single nerve cell apoptosis in experimental neurodegeneration, we are also keen to assess its use in combination with other spectrally distinct cell markers. This should permit investigation of fundamental disease mechanisms and the evaluation of interventions with clinical applications. Furthermore, as we and others have advocated, as the retina is increasingly implicated in a variety of neurodegenerative conditions,11 we believe that investigation of such mechanisms within the eye may shed light on mechanisms underlying neurodegeneration within the brain.

DARC may thus provide a powerful new clinical tool with which to diagnose and identify patients with early glaucoma, before they lose vision. It may also dramatically reduce the duration of glaucoma clinical studies, which currently have to use visual field status as a key end-point and determinant of outcome. In clinics, it could provide a real-time, more rapid and objective method by which to monitor patients. Finally, it may also serve as a new method of assessing central nervous system (CNS) degeneration.

As we await the results of the phase 1 clinical trial at the Western Eye Hospital in London, we all hope that DARC may provide the new end-point that we so clearly need in glaucoma.

**Table 1: Rate of Retinal Ganglion Cell Loss and Apoptosis in Hypothetical Glaucoma Patient**

<table>
<thead>
<tr>
<th>Hypothetical Patient Age (years)*</th>
<th>50</th>
<th>51</th>
<th>52</th>
<th>53</th>
<th>55</th>
<th>60</th>
<th>70</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal RGC loss/year (x 10³)</td>
<td>1,000</td>
<td>996</td>
<td>992</td>
<td>988</td>
<td>980</td>
<td>961</td>
<td>923</td>
<td>887</td>
</tr>
<tr>
<td>Glaucoma RGC loss/year (x 10³)</td>
<td>1,000</td>
<td>960</td>
<td>922</td>
<td>885</td>
<td>815</td>
<td>665</td>
<td>442</td>
<td>290</td>
</tr>
<tr>
<td>Glaucoma RGC apoptosis/year</td>
<td>3,000</td>
<td>10,934</td>
<td>142,265</td>
<td>114,251</td>
<td>54,524</td>
<td>12,964</td>
<td>8,619</td>
<td>5,730</td>
</tr>
<tr>
<td>Glaucoma RGC apoptosis/day</td>
<td>8</td>
<td>50</td>
<td>200</td>
<td>400</td>
<td>600</td>
<td>1400</td>
<td>5000</td>
<td>5000</td>
</tr>
</tbody>
</table>

*Glaucoma onset at 50 years of age.

The rate of retinal ganglion cell (RGC) loss in glaucoma is predicted to change from 0.4% (normal age-related loss) to 4% per year (glaucomatous loss), as previously described by Zeyen. RGC apoptosis numbers have been calculated using these rates, but also taking into account the extrapolated profile of RGC apoptosis shown in Figure 2, in a hypothetical clinical situation of a patient with sudden onset of glaucoma at 50 years of age with the same profile of RGC apoptosis as the rat ocular hypertension model. Predicted levels of RGC apoptosis per year and per day are calculated, as shown.

**Figure 3: Predicted DARC Count in Hypothetical Glaucoma Patient**

This graphical display of data from Table 1 shows the predicted DARC count (retinal ganglion cell (RGC) apoptosis count/day) in the hypothetical clinical situation of a patient with sudden onset of glaucoma at 50 years of age. The maximal expected DARC count occurs within 10 years of the onset of disease (shaded area), and coincides exactly with the time-tag currently estimated as the delay in visual field perimetry detecting abnormalities.15

![Image](http://example.com/darc-count-graph.png)
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