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DARC as a potential surrogate marker

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Abstract

Glaucoma is a progressive, neurodegenerative disease that is increasing in prevalence worldwide. There is a need to develop ways in which to diagnose the disease sooner and more reliably, in order to prevent irreversible visual loss and meet the growing demands on healthcare services. Research into neuroprotective therapies in glaucoma is lacking a reliable surrogate marker in order to show treatment efficacy in a meaningful and cost-effective manner. The Detection of Apoptosing Retinal Cells (DARC) is a new technique that has promise in providing a solution to this unmet clinical need. Multiple animal studies have demonstrated its use as a biomarker in quantifying the effect of retinal neuroprotection methods, and it has recently been translated into humans in Phase I and II trials, with Phase I demonstrating the visualisation of individual apoptosing retinal cells in healthy and glaucomatous patients, with good safety and tolerability. The future for this technique will now be identifying disease-specific characteristics of human disease that can be used in order to provide us with a much-needed surrogate marker in the field of retinal neurodegeneration.

Introduction

Glaucoma is the leading cause of irreversible blindness affecting more than 70 million people worldwide[1]. Its physiopathologic process results in the excavation ("cupping") of the optic nerve and irreversible visual loss if the disease is not detected early. Whilst it is common for this disease to progress slowly over many years, this also makes detection of visual field changes difficult to detect over short periods of time in order to make the diagnosis. This problem demonstrates the necessity to develop more surrogate markers which can accurately predict future vision loss. A surrogate marker is defined by its ability to predict a clinically relevant outcome such as vision loss or decrease in quality of life, as well as correlating with the disease process. In addition, the effect of a treatment on the surrogate must have an effect on the clinical outcome[2]. Their predictive power not only allows earlier diagnoses, but also affords shorter, and therefore more economical clinical trials and thus are an attractive prospect from many perspectives[2].

Presently, the most important risk factor for glaucoma is raised intraocular pressure (IOP). The Ocular Hypertension Treatment Study (OHTS) has shown that an increased IOP of one mmHg is associated with a 10 to 25% increased risk of disease progression[3]. Although IOP is accepted as an important risk factor, its use as a surrogate has never been properly validated[4] as IOP does not always correlate with disease. For instance, patients with normotensive glaucoma (NTG) have an IOP within 'normal' limits despite having progression of disease[5]. Alternatively, some patients with ocular hypertension do not experience any visual field loss[4, 6]. Therefore, interventions such as surgery to lower IOP may not provide any benefit to

some patients, whilst still exposing them to the risks of the treatment. In the Low-pressure Glaucoma Treatment Study (LoGTS), patients were treated either with timolol or brimonidine. Both treatments showed a similar reduction in IOP but brimonidine was more effective at protecting from progressing visual field defects[7]. By not 'capturing' the extra beneficial treatment effect in the brimonidine group, the results suggest that IOP may be deficient as a surrogate marker for glaucomatous progression.

With the emergence of new imaging techniques during the last decade, the use of imaging of structural biomarkers as a surrogate has been investigated. The clear structure-function relationship between visual field defects and visible changes in the optic nerve gives more comprehensive information on disease progression than IOP[8]. Indeed, some studies have shown that progressive changes in imaging of the retinal nerve fibre layer (RNFL) thickness is predictive of visual field loss over 5 years[9]. However, there lacks a strong correlation between these parameters measured at a single visit and future visual field loss[4]. With validation of functional endpoints, structural surrogates may prove a better biomarker for the assessment of neuroprotective therapies, as their primary goal is not to lower the IOP. This validation with functional endpoints is required in order to avoid endorsing novel therapies that have a positive effect on a structural parameter, but provide no benefit to visual function.

Principles of DARC

Annexin-A5 is an endogenous ubiquitous protein present in animals and humans. It has the ability to bind to phosphatidylserine (PS) which is an early marker of cell apoptosis[10] . PS is normally found on the internal leaflet of the cell membrane, however when a cell is in the early stages of apoptosis, PS flips to the exterior leaflet of the cell membrane allowing annexin to bind it in the presence of calcium[10].

Annexin V has been widely used in research during the last decade. Animal studies using radiolabelled annexin in combination with positron emission tomography (PET) or a single-photon emission computed tomography (SPECT) scan have shown an increased uptake of annexin in areas where apoptosis occurs[11]. Human studies have also been performed in relation to myocardial infarction [12], cardiac tumours[13], cardiac allograft rejection [14] and neck and head carcinomas [15]. All these studies showed an accumulation of radiolabelled annexin in the areas of interest. However, the resolution of these techniques is limited by the opacity of these organs and often deep location within the body.

Due to the transparency of ocular media, the eye is a unique organ which permits direct visualisation of retinal cells. The Detection of Apoptosing Retinal Cells (DARC) is a technique using modified confocal scanning laser ophthalmoscopy (cSLO) which takes advantage of these properties of the eye in order to allow real-time visualisation of apoptotic retinal cells at a single cell level, using fluorescently labelled annexin-A5 [16] (figure 1).

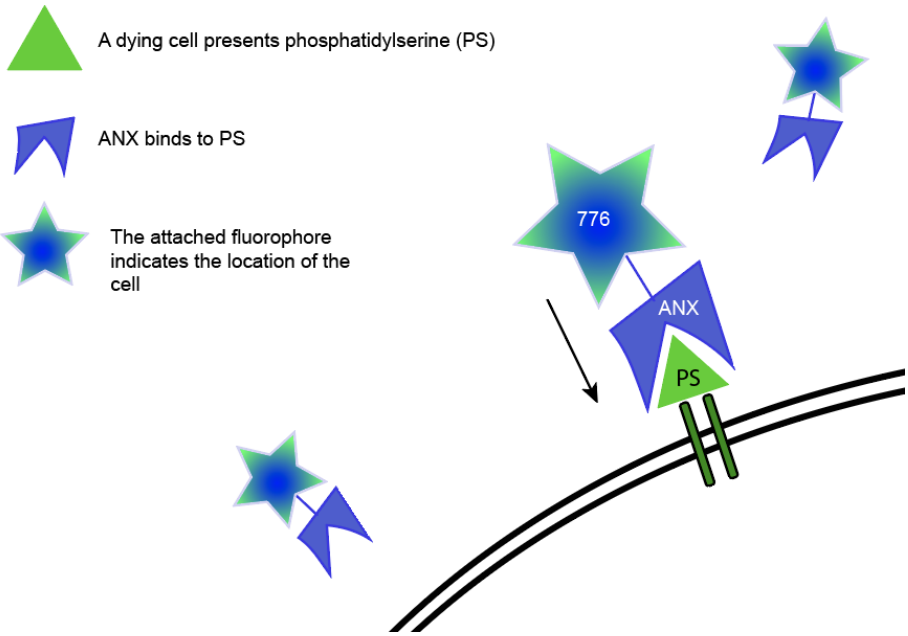

Figure 1 Diagrammatic representation of DARC technology. Annexin-A5 (ANX) binds to phosphatidylserine (PS) on the outer leaflet of cells in early apoptosis. The attached fluorophore is then detected using scanning laser ophthalmoscopy.

In vivo experimental studies using DARC to date

Since the DARC technique was established in 2004[16], we have used various experimental animal models for characterization of the natural history of disease and assessment of neuroprotective agents. Retinal ganglion cell apoptosis occurs not only in ocular disease, such as glaucoma and other optic neuropathies, but also neurological disorders, including Alzheimer's disease (AD) [17-19] and Parkinson's disease (PD)[20]. DARC imaging has been demonstrated to be a useful tool for screening drug efficacy in those models [20 21 22-24]. In this review, we only focus on DARC applied to therapeutic strategies in disease models.

Glaucoma-related models can be induced either surgically or chemically to cause RGC apoptosis. To evaluate DARC sensitivity and accuracy in monitoring RGC apoptosis, we firstly assessed its performance in well-established rat models of chronic ocular hypertension (OHT) and optic nerve transaction (ONT)[16], and found RGC apoptosis was occurring over time and peaked at 3 weeks in OHT and 7 days in ONT models. At the end of experiments, RGC apoptosis accounted for a total RGC loss of 60% at 16 weeks in OHT and 76% at 12 days in ONT models. Using DARC we also detected RGC apoptosis in a mouse model following intravitreal administration of staurosporine (SSP)[25].

Glutamate excitotoxicity has been implicated in RGC death in glaucoma[25-27], and blocking NMDA receptors by specific antagonists, such as memantine, an uncompetitive NMDA antagonist and the best-known glutamate modifier in Alzheimer's disease treatment [28], was reported to be effective in reducing RGC

death in experimental glaucoma[29-31]. Using DARC, we have firstly assessed the effects of different glutamate modulation strategies, including a nonselective (MK801) and a selective (ifenprodil) NMDA receptor antagonists and a metabotropic glutamate receptor agonist (mGluR Group II, LY354740), in an SSP-induced rat model of RGC apoptosis[22]. We found that all three single agents significantly reduced RGC apoptosis in a dose-dependent manner, but combining low-dose MK801 with LY354740 appeared to be most effective compared to either agent alone. We then applied the most optimal combination regimens to the OHT model at different time points (0, 1 and 2 weeks following IOP elevation). DARC results revealed the most effective timing of treatment was at 0 weeks (the time of IOP elevation). We believe this is due to maximal inhibition of glutamate release after primary insult, resulting in reduction of secondary degeneration that can lead to neuronal degeneration far beyond the primary injury site[32].

Amyloid-ß (Aß) plaques in the brain are a hallmark in Alzheimer's disease, with Aß deposition also found to be implicated in the development of RGC apoptosis in glaucoma by us and others[23, 33, 34]. Aß is derived from the proteolytic cleavage of amyloid precursor protein (APP). Two catabolic pathways are identified for APP processing. One is the nonamyloidogenic pathway via α-secretase activation, resulting in secretion of soluble forms of APP (sAPPα), and the other is the amyloidogenic pathway, where ß- and ϒ- secretase activation leads to Aß generation. Using DARC, we have assessed the effects of targeting the amyloidogenic pathway on RGC protection in the OHT rat model, by examining three different agents: an anti-Aß antibody (Aßab), a ß-secretase inhibitor (ßSI) and Congo red (CR). CR is a dye commonly used to stain amyloid-ß histologically and has been

shown to block Aß aggregation [35, 36]. Our DARC data showed that all three single agents altered the profile of RGC apoptosis in a temporal manner by delaying the development of peak RGC apoptosis and reducing peak levels. However, although anti-Aß ab appeared to be more effective in prevention of RGC apoptosis than the other two agents, a combination of three agents demonstrated to be the best regimen, resulting in the maximal reduction of RGC apoptosis[23].

Modulation of the nonamyloidogenic pathway has also been investigated in the OHT rat model. Brimonidine (BMD) and clonidine (Clo) are α2 adrenergic receptor agonists (α2ARAs), drugs used to lower intraocular pressure in glaucoma patients [37]. Both BMD and Clo have been reported to be neuroprotective[38-40]. Using DARC, we have recently demonstrated that systemic administration of α2A agonists BMD and Clo significantly reduced ocular hypertension-induced RGC apoptosis in vivo. Our results revealed that the protective effect of α2A agonists was associated with reduced levels of Aß deposition in retinal ganglion cell layers, where an increase of Aß immunostaining was found in the ocular hypertension model[23, 37]. In this study, we also found that α2ARAs modulated the levels of laminin and MMP-9 (metalloproteinase-9), potentially linked to changes in Aß through APP processing, promoting the non-amyloidogenic pathway.

Mitochondrial dysfunction and oxidative stress mediate RGC death in glaucoma [41, 42]. Coenzyme Q10 (CoQ10) is a mitochondrial targeted antioxidant and plays an essential role in the normal function of the electron transport chain. CoQ10 exhibits neuroprotection in neurological disorders such as AD, PD, and Huntington's disease (HD)[42], and also experimental glaucoma[43]. Using DARC, we have recently evaluated

topical administration of CoQ10/TPGS (α-tocopherol polyethylene glyucol succinate) micelles in the OHT rat model[44]. DARC data demonstrated that topical CoQ10 treatment significantly reduced OHT-induced RGC apoptosis compared to vehicle controls. DARC results are consistent with retinal whole-mount histology outcomes, in which topical CoQ10/TPGS but not TPGS controls can protect Brn3a+ RGCs against apoptosis as indicated by the preservation in RGC density and nearest neighbour distance.

Optic neuropathy describes a collection of disorders characterised by damage to the optic nerve and loss of RGCs due to any cause including glaucoma, ischaemia, trauma and genetic predisposition. Experimental models of optic neuropathy can be made by transection of the optic nerve fully (ONT) or partially (pONT)[45]. The pONT model represents a reliable and reproducible model for studying secondary degeneration, a phenomenon believed to occur in the central (CNS) and peripheral (PNS) nervous systems, where injury from initial lesions can lead to widespread damage to neurons beyond the primary injury site. We have used the pONT model to assess therapeutic effects on secondary degeneration[45].

Cell-based therapies are increasingly recognized as a potential strategy to treat retinal neurodegenerative disease. Their administration, however, is normally indirect and complex, often with an inability to assess in real-time their effects on cell death and their migration and integration into the host retina. Using DARC to monitor RGC apoptosis, we have assessed a novel delivery route, i.e. direct optic nerve sheath (DONS) application of Schwann cells (SC) in a pONT rat model[45]. DARC data showed that the DONS application of Schwann cells significantly reduced pONT-

induced RGC apoptosis at 7 and 21 days, compared to untreated controls. The DARC results were comparable to histological findings, where SC/DONS therapy significantly increased Brn3a+ RGC survival in retinal whole-mounts, by mostly targeting secondary degeneration.

Adenosine is a neuromodulator in the CNS, with its biological effectsmediated through G protein-coupled receptors. The adenosine A3 receptor (A3R) is believed to be involved in a variety of different intracellular signalling pathways. RGCs express A3R, and activation of A3R is neuroprotective. Using DARC, we have assessed the effects of intravitreal administration of A3R against RGC apoptosis on the pONT model. We found that A3R significantly reduced number of apoptotic RGCs compared to the non-treatment group. The in vivo findings were consistent with histological observations of reduced Brn3a+ RGC loss[46].

Parkinson's disease (PD) is an important cause of dementia worldwide, however there are currently no effective biomarkers for early diagnosis and no effective treatments for altering disease progression. Although it is a neurological disorder, mounting evidence shows that the eye, particularly the retina, is also affected[47]. Using a rotenone-induced rat model of PD, we have assessed therapeutic strategy by DARC imaging. We have firstly evaluated retinal changes by longitudinal imaging of RGC apoptosis with DARC and retinal thickness with OCT (optical coherence tomography), and compared it with brain histology. The imaging data revealed that RGC apoptosis significantly increases as early as day 20 of a rotenone insult, accompanied by a transient swelling of retinal layers. However, characteristic histological neurodegenerative changes in the substantia nigra and striatum occur

from day 60, suggesting that retinal changes precede the pathological manifestations of PD.

We then evaluated therapeutic effects of systemic administration of different formulations of rosiglitazone by in vivo imaging, and found sustained release administration of liposome-encapsulated rosiglitazone to be the most effective among the treatment regimens tested, as evidenced by significant reduction of retinal neuron apoptosis at day 20, and nigrostriatal neurons at day 60. This is the first in vivo evidence of RGC loss and early retinal thickness alterations in a PD model, as well as therapeutic assessment in the model. This would suggest that retinal changes may be a good surrogate biomarker for PD, which may be used to assess new treatments clinically.

DARC in clinical trials

The challenge following pre-clinical studies was to reproduce DARC technology in human subjects in order to examine its potential as a clinically useful research or diagnostic tool. DARC has now been trailed in humans, with encouraging results. ANX-776 using the human-variant annexin molecule, RhAnnexin V128, was the fluorescently-labelled annexin taken forward to clinical trials. With excitation and emission wavelengths of 771nm and 793nm respectively, this near infrared fluorophore lends itself well to clinical translation, being compatible with the 786nm diode laser excitation and 800nm barrier filter used in indocyanine green (ICG) imaging when examining choroidal pathology, such as in idiopathic polypoidal choroidal vasculopathy (IPCV)[48].

The Phase I clinical trial of DARC set out to establish the safety and tolerability of the technique in humans using a Storer design[49], enrolling 8 progressing glaucoma patients and 8 healthy volunteer controls. Glaucoma patients were deemed to be progressing based on one mode of investigation including visual field testing, OCT scans and Heidelberg Retinal Tomography (HRT). 2 glaucoma patients and 2 healthy controls were randomly allocated to a 0.1mg, 0.2mg, 0.4mg 0.5mg intravenous dose of ANX-776. The intravenous agent was well tolerated with no serious adverse events, and no adverse events suspected to be in relation to the ANX776 itself. Pharmacokinetic analysis was conducted with peripheral blood sampling at 5, 15, 30, 60, 120 and 300 minutes post-administration. This showed that ANX-776 was rapidly absorbed and eliminated with dose-dependent levels in the blood and no accumulation. During the course of the trial and up to 30 days post-

administration, no patients withdrew from the study and there were no serious adverse events recorded. Six adverse events were reported including discomfort and bruising due to phlebotomy, dizziness, headache, influenza and metatarsal inflammation, all of which were self-limiting and none deemed to be related to the ANX-776. A key finding was there was a significant measure of efficacy, both with respect to DARC counts being higher in glaucoma patients compared to healthy controls, but also with respect to predicting future glaucoma disease progression – in fact 18 months before changes in OCT and visual fields. This latter finding suggests that DARC could be a potential surrogate marker.

The Phase II clinical trial of DARC in process was designed to fully characterise the differences between the counts and distribution of DARC spots in health and disease. Patients (116) have been recruited into 5 different groups including healthy volunteers, progressing glaucoma, age-related macular degeneration, optic neuritis and Down's syndrome, (in whom CNS pathology similar to Alzheimer's disease is found[50].) The results of this trial are to be published soon.

The impact of DARC

There is a clear need for more sensitive biomarkers in the fields of glaucoma and neurodegeneration that will allow us to detect disease earlier, and therefore instigate treatment. This is especially pertinent in neurodegenerative conditions such as glaucoma whereby late detection may miss the considerable amount of damage that can occur prior to clinically detectable changes in visual fields[51-53]. Furthermore, the theory that cells may have a lower resistance to injury following primary insults[54] would also support early detection to prevent further cell death and subsequent secondary degeneration. Thus, instigating treatments later in the disease course is likely to result in poorer clinical outcomes and reduced observed treatment efficacy, and therefore is often given as a possible explanation for the failure of novel therapeutics in the field of neuroprotective research, as indeed is the sensitivity of diagnostic techniques used in such trials[55].

In contrast to the currently used diagnostic techniques which observe structural or functional damage that has already occurred, DARC holds the potential to observe cellular apoptotic events in real-time, which may later contribute to the changes seen in OCT parameters and visual field tests. Clinically this has the potential to reduce morbidity from visual field loss, and the ability to conduct neuroprotection clinical trials in a faster, more cost-effective manner, whilst testing treatments in an IOP-independent manner. Further trials are needed to validate these early findings.

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