Detecting apoptosis as a clinical endpoint for proof of clinical principle

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Abstract

The transparent eye media represent a window through which to observe changes occurring in the retina during pathological processes. In contrast to imaging the extent of neurodegenerative damage that has already occurred, imaging an active process such as apoptosis has the potential to report on disease progression and therefore the threat of irreversible functional loss in various eye and brain diseases. Early diagnosis in these conditions is an important unmet clinical need to avoid or delay irreversible sight loss. In this setting, apoptosis detection is a promising strategy with which to diagnose, provide prognosis, and monitor therapeutic response. Additionally, monitoring apoptosis *in vitro* and *in vivo* has been shown to be valuable for drug development in order to assess the efficacy of novel therapeutic strategies both in the pre-clinical and clinical setting. Detection of Apoptosing Retinal Cells (DARC) technology is to date the only tool of its kind to have been tested in clinical trials, with other new imaging techniques under investigation in the fields of neuroscience, ophthalmology and drug development. We summarize the transitioning of techniques detecting apoptosis from bench to bedside, along with the future possibilities they encase.

Introduction

Clinical relevance of apoptosis detection

The eye represents a privileged window through which we can view the central nervous system, offering clinicians and researchers the opportunity to use retinal biomarkers in the diagnosis and monitoring of neuronal physiology and pathology in-vivo. Apoptosis of retinal cells is the common endpoint of different insults occurring in a variety of neurodegenerative diseases(1). The archetypal neurodegenerative disease of the retina is glaucoma, characterised by retinal ganglion cell (RGC) apoptosis, although other modes of death have been proposed(2,3). Accompanying RGC loss are retinal nerve fibre layer (RNFL) thinning, optic disc cupping and irreversible loss of visual field which can all be clinically detected (4). Pathological death of RGCs has also been detected in neurodegenerative conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), optic neuritis and multiple sclerosis. In contrast, other ophthalmic conditions may involve different cell populations; for example, in age-related macular degeneration (AMD), retinal pigment epithelium and photoreceptors progressively degenerate, leading to central vision loss (5). Monitoring the rate of this underlying degenerative process is of great importance in order to guide treatment and indicate prognosis.

Retinal ganglion cell (RGC) loss is a physiological process ubiquitously occurring due to ageing; however, the progression rate of RGC loss is significantly higher in subjects affected by glaucoma (6). We usually lose approximately 0.4 % of our RGC population per year, while in glaucomatous patients the rate is increased approximately 10-fold (6,7). On average, a healthy subject has around 1.2 million RGC at birth(6), with approximately 20-40% thought to be lost before visual field defects are detected(8). This leads to a diagnostic delay of up to ten years (9). Tools to measure the rate of programmed cell death in a minimally-invasive manner will hopefully complete the standard eye examination of the future, if they can replace the need for extended follow up that is responsible for the delay in many diagnoses. Moreover, repeated measures of apoptosis detection will hopefully provide an IOP-independent, and robust clinical trial biomarker.

Apoptosis within the glaucoma paradigm

The most widely accepted and only currently modifiable factor associated with glaucoma is elevated intraocular pressure (IOP) (10,11). The axons of RGCs gather at the optic disc and cross the sclera at the level of a structure known as the lamina cribrosa. IOP may cause excessive stress on RGC fibers at this level, interrupting the orthograde and retrograde axonal trafficking of nutrients and trophic factors (12). Moreover, during the periods of elevated IOP, metabolic stress is induced and energy demands of RGC and astrocytes rise, leading to mitochondrial dysfunction (13). However, raised IOP is neither necessary nor sufficient in isolation for diagnosis (14). It is the characteristic RGC death that defines the disease, heralded by apoptosis occurring in order to avoid a destructive localized or systemic inflammatory reaction. Apoptosis-initiating events (such as raised IOP) are followed by cell shrinkage and blebbing, chromatin condensation and DNA fragmentation (15), but a very early event in this process is the translocation of phosphatidylserine (PS) to the external leaflet of the cell membrane (16); this can be exploited by in vitro and in-vivo diagnostic techniques.

Retinal cell apoptosis in other eye and brain diseases

A deregulated programmed cell death is thought to occur in other retinal diseases such as age-related macular degeneration (AMD) (17), diabetic retinopathy (DR), and other retinal dystrophies. In all these conditions, monitoring apoptosis may represent a surrogate biomarker of disease activity and progression. AMD is the leading cause of irreversible blindness in the ageing population (18), and a major worldwide health problem. The primary insult in AMD occurs at the level of RPE, due to accumulation of yellowish autofluorescent lipofuscin deposits above Bruch's membrane and beneath RPE cells, known as drusen. Drusen are responsible for the distortion of central vision in ‘dry’ AMD, the size of which may range from tiny dots up to 250µm (18). Larger drusen have a tendency to fuse leading to pigment epithelial detachment (PED). This last phenomenon represents a risk factor for the development of the 'wet' form of AMD, during which neovascularisation from the choroidal circulation causes exudation and haemorrhage destructive to the anatomical order of the retinal layers. Secondary to RPE degeneration, rod and cone dysfunction also accounts for central vision loss, a characteristic feature of the pathology (18). Notably, rods are more severely affected by AMD (19), with significant rates of photoreceptor and RPE apoptosis seen with TUNEL staining (Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling) (20).

Diabetic retinopathy (DR) is a common complication of diabetes mellitus. It is the most common cause of vision loss among amongst working age groups and represents a huge socio-economic burden (21). The most striking pathological changes of DR are the microvascular complications

occurring in the retinal tissue; however, DR also involves an increased rate of apoptosis both in vascular and neuro-retinal cells as shown by TUNEL assay based studies (22). The cell populations mostly involved in this phenomenon are RGCs and amacrine cells (23).

The degeneration of some retinal cells populations has been associated with brain disorders such as AD and PD (24). AD is by far the most common form of dementia, accounting for approximately 70 per cent of all cases(25). The main pathological and diagnostic feature is represented by the deposition of extracellular senile plaques and intracellular neurofibrillary tangles. Nowadays, diagnosis is primarily based on the patient's behavioural and clinical assessment (26) and, secondarily, confirmed by either computed tomography or magnetic resonance imaging (27). The problem of this current approach is related to the irreversibility of the damage at the time of diagnosis, while by contrast, the pathological accumulation of amyloid may have begun 10 to 15 years earlier (25). This underlines the need for novel strategies for early disease detection and prevention. The idea of using the eye as a window over the central nervous system (CNS) for diagnosis of AD is rapidly growing since retinal nerve fibre layer loss has been widely observed (1). The detection of beta amyloid in the retina of Alzheimer's patients has produced conflicting results (1). In AD, PD, HD and glaucoma there are common elements such as oxidative stress, mitochondrial dysfunction, excitotoxicity and misfolded protein aggregation. Therefore, there is potential for apoptosis detection as a biomarker of dis ease diagnosis in all of these diseases(28).

In vivo Apoptosis detection - Detection of Apoptosing Retinal Cells (DARC)

Apoptosis detection *in-vivo* exploits modifications of apoptotic cells undergoing programmed cell death: phosphatidylserine (PS) exposure, changes in apoptotic membrane imprint and caspase activation.

Since its first description in 2004, DARC technology has been used for the assessment and follow-up of different animal models of degenerative retinal diseases for natural history characterization and the study of novel neuroprotective agents (29). Moreover, ongoing clinical trials are evaluating DARC performance in human subjects.

The principle of DARC is based on the use of fluorescently-labelled annexin A5. Although ubiquitously expressed, annexin A5's function is not fully understood. However, its ability to bind PS in a calcium-dependent manner is exploited in apoptosis detection (30). The majority of PS is usually maintained on the intracellular aspect of the cytoplasmic membrane due to the action of ATP-dependent

'flippases'; however, increasing translocation to the extracellular surface occurs during cell stress and the process initiating apoptotic cell death, possibly with the involvement of 'scramblases' (31). PS represents an 'eat me' signal for phagocytes, removing apoptotic debris to prevent pro-inflammatory consequences (32).

Annexin A5 was first developed for *in-vitro* labelling of apoptosis but was soon re-developed for *in-vivo* imaging of apoptosing tissues using radioactive tracers in combination with positron emission tomography (PET) and single-photon emission computed tomography (SPECT) nuclear imaging techniques (33). The main applications of this type of studies related to oncology, inflammatory bowel diseases, myocardial infarction and strokes(34–36). For DARC to accomplish retinal imaging, annexin A5 has been fluorescently labelled using both a 488nm, and near-infrared 776nm tags with excitation and emission spectra of 495-519 nm and 771-793 nm respectively (ANX776) (37).

Modified confocal scanning laser ophthalmoscopy (cSLO) is used to image the retina using the in-built fluorescent detection systems, providing high-contrast retinal images (38). The latter near-infrared wavelength of ANX776 is aligned to that of indocyanine green (ICG) for which imaging set-ups are in widespread use by medical retina specialists. The field of image acquisition is between 30-55° and can be either centered on the fovea or the optic disc. Compensation for non-enhancing structures and non-linear distortion is performed post-acquisition processing (39), with quantification of apoptosing cells performed via a template-matching approach to count hyperfluorescent spots, a count known as the 'DARC count' (40).

DARC technology in pre-clinical and clinical studies

Pre-clinical studies using DARC

DARC has been used in many pre-clinical studies including animal models of glaucoma and other neurodegenerative conditions to investigate disease pathogenesis and the effectiveness of potential treatments. The first study was published in 2004 demonstrating histological validation and disease activity in a rat model of glaucoma (29). DARC has also been used to characterize the relationship between raised IOP and retinal apoptosis, an initiating injury used in many animal models of glaucoma (41). Following on from this work, a novel staurosporine-induced rat ocular hypertension model was demonstrated and used to investigate the neuroprotective effect of modulating glutamate-induced excitotoxicity (42). DARC was used to investigate the role of amyloid plaques in retinal tissue and their relationship with retinal apoptosis. These deposits were related to the rate of

apoptosis in a dose- and time-dependent manner, with strategies to prevent amyloid plaque formation or enhancement of their clearance, beneficial in terms of RGC survival (43).

Retinal apoptosis has also been monitored in diabetic mice, which had increased DARC counts in comparison to wild-type controls. These results support the use of in-vivo apoptosis detection as an early biomarker of DR, before visible vascular changes are detectable on fundus examination (44).

Photoreceptor loss whilst investigating the role of blue light exposure in dark Agouti rats has been characterised by DARC, revealing hyperfluorescent apoptotic cells in the outer retina, confirmed by histological staining of photoreceptors (45). This last study prompted further investigation into macular degeneration, where DARC was able to detect the presence of apoptosis in photoreceptors in a mouse model of dry AMD (18).

DARC has been used to investigate the potential of novel therapeutics such as the effectiveness of MRZ-99030, a modulator of amyloid-beta aggregation, as a neuroprotector. The study highlighted a dose-dependent reduction of apoptosis upon systemic injection of the molecule (46). Another study used the partial optic nerve transection (pONT) model to show the ability of 2-Cl-IB-MECA, an adenosine A3 agonist, to reduce retinal apoptosis *in-vivo* (47). Brimonidine, an alpha-2 adrenergic receptor agonist, was shown to be able to reduce the rate of retinal cell apoptosis through an IOP-independent mechanism related to amyloid precursor protein aggregation modulation (48). Coenzyme Q10 was also shown to facilitate significant reduction of retinal apoptosis in-vivo using DARC(49). A liposomal formulation of rosiglitazone, a peroxisome proliferator-activated receptor-gamma agonist, was used with DARC in a rotenone-induced rat model of PD (50). The results of the study showed protection both at the level of the retina with reduced retinal apoptosis, and in the nigrostriatal pathways of the brain (50). More recently, topical nanoparticles of memantine, an NMDA receptor antagonist used to treat AD, and curcumin, a naturally occurring polyphenol found in turmeric, have been tested for their neuroprotective abilities in vivo (51,52). DARC demonstrated both were able to provide significant reduction in retinal apoptosis in rat models of ocular hypertension (51,52). A novel cell-based therapy, the delivery of Schwann cells directly on the damaged optic nerve sheath, was also shown to produce sustained results promoting axon regrowth and preventing secondary RGC neurodegeneration using DARC (53).

DARC in the clinical setting

Phase I clinical trial

After the promising results in animal models, DARC technology has been tested in the clinical setting, with the phase I clinical trial published in 2017(37). This was a single-centre, open-label, proof-of-concept clinical trial designed to primarily assessing safety, and secondarily, efficacy of DARC imaging in humans (37). The study was carried out on eight healthy volunteers and eight patients affected by progressing glaucoma. These subjects were randomly allocated to one of the different ANX776 dosage groups. Each of the four dosage groups included two glaucoma patients and two healthy controls. After the single intravenous injection of ANX776, retinal imaging was performed to visualize fluorescently labelled retinal cells at 15, 30, 60, 120, 240 and 360 minutes (Figure 2). Apoptotic retinal cells were identified as hyperfluorescent areas on the retina of a size between 12 and 16 µm using a cSLO focused on the RGC layer. DARC spots were objectively counted using a method of template matching to track them longitudinally (40,54).

All subjects were required to attend three visits, and a follow up at 30 days. They underwent standard eye examination, including best-corrected visual acuity, tonometry, gonioscopy, dilated fundus examination, optical coherence tomography (OCT) and standard automated perimetry. Additionally, all glaucoma patients were regularly followed as part of the standard glaucoma care up to 16 months after DARC. This strategy allowed the investigators to track glaucoma progression and compare the standard indices with DARC, in order to test the potential of DARC as a predictive surrogate marker.

No patients were withdrawn from the study, and no serious adverse events occurred. The study reported only isolated cases of discomfort during phlebotomy, hematoma at cannulation site, influenza, metatarsal inflammation and dizziness. ANX776 showed rapid absorption and elimination without accumulation. The half-life of the drug ranged from 18 to 36 minutes, with maximal concentration proportional to the dose administered. These results were consistent with other studies using radio-labelled annexin A5 (55).

The greatest difference between healthy controls and glaucoma patients was seen when ANX776 was administered at a dose of 0.4 mg (p-value < 0.01). Multivariate analysis showed a 2.4 fold higher DARC count in glaucoma patients across the 6 hours monitored (95% confidence interval (CI):1.4-4.03; p=0.003). DARC count was found to be significantly correlated with decreased central corneal thickness, increased cup-disc ratio and increased age. Post hoc it was shown that DARC was able to

predict the increased rate of progression, therefore, showing the potential prognostic role of this technology.

Overall, this study proves the safety of the intravenous administration of ANX776 in human subjects and suggests the optimal dosage for apoptosis detection. The results suggest DARC technology may have clinical potential for early glaucoma diagnosis, and monitoring for progression and therapeutic success.

Phase II clinical trial

Following the successful results of the phase I clinical trial, DARC imaging technology has undergone phase II clinical evaluation, the results of which are due to be published shortly. It is a single-centre, non-randomised, open-label clinical trial examining the use of ANX766 to image retinal apoptosis in the retinas of healthy volunteers, patients (n=116) affected by glaucoma, AMD, optic neuritis and Down's syndrome (with pathology similar to Alzheimer's disease (56)). The patients enrolled for this study received a single shot of ANX776 at a dose of 0.4 mg and then were imaged at 15mins, 2 and 4 hours after the injection. This primary objective of this study is to assess the DARC count in different pathologies, and further assess DARC's potential in early diagnosis and predictive abilities.

PSVue 550

PSVue550 (Bis(zinc(II)-dipicolylamine, Zn-DPA) is a synthetic molecule able to bind to PS, conjugated with a fluorophore known as Texas red. The affinity of this probe for PS makes it suitable to transiently visualise apoptosis in the retina, allowing for repeated fluorescent imaging. This molecule has shown efficacy with topical administration in rat and mice models of photoreceptor diseases (57), with no direct retinal toxicity of the probe(57).

In the experiment conducted by Mazzoni et al. eye penetration of PSVue550 was tested on Royal College of Surgeons (RCS) rats, a well-characterised model of retinal degeneration, and wild-type controls. They were able to show that irrespective on retinal degeneration the dye was able to reach the posterior segment of the eye. However, only apoptotic photoreceptors of RCS rats could be visualised (57). They tested the penetration of another annexin derived molecule able to tag PS, Polarity Sensitive Indicator of Viability and Apoptosis (pSIVA). It was shown a similar fluorescence pattern upon intravitreal injection of either PSVue550 or pSIVA; however, only PSVue550 was able to reach posterior retina upon topical application. These results were obtained upon histologic sample examination fluorescence microscopy (57).

PSVue550 toxicity was tested through photoscopic full-field electroretinograms on dark-adapted rats at three days after eye drop subministration. Control RCS rats received topical Hanks buffered saline solution. No statistical difference to light response could be detected between the treated and control group(57).

Additionally, live imaging of apoptotic photoreceptors in vivo by whole animal scanning was performed in order to assess whether labelling of apoptotic cells was permanent or transient. The results showed that the peak fluorescence could be detected at 24 hours after topical subministration. Moreover, after 72 hours, there was no statistical difference in the level of fluorescence of treated and control eyes. Therefore, the transitory nature of PSVue550 labelling has the potential to be exploited for serial monitoring of retinal degeneration at different time points. Using this imaging technique, they validated the results also obtained in other mice models of degenerative photoreceptor disease such as the MerTK-deficient model and the wild-type rat with light-induced retinal damage model (57).

To further validate this approach, live imaging of apoptotic photoreceptors through retinal imaging was performed. They were able to show a statistically significant difference in retinal fluorescence in treated versus control eyes (57). The safety and utility of this approach in human subjects are yet to be reported.

Apoptotic membrane imprint modification

ApoSense®

Aposense® is a molecular imaging technique using amphipathic low-molecular weight molecules of 300 to 700 Daltons. These selectively cross the apoptotic plasma membrane and accumulate in the cytoplasm of dying cells (58). The hydrophobic region enables the anchoring of these molecules to the lipidic surface of the cell membrane, whilst the hydrophilic region would usually block their entrance into the cytoplasm of non-apoptotic cells. Their accumulation in the cytoplasm has been shown to occur alongside recognized apoptotic events such as PS exposure, caspase activation and the loss of mitochondrial membrane potentials. These compounds can be labelled or rely on intrinsic fluorescence, or undergo labelling with a radioactive moiety. Molecules belonging to this family include N,N'- didansyl-L-cystine (DDC), NST-732 and 729, ML-9 and ML-10. The first three contain a dansyl group, while the last two contain an alkyl-malonate molecule(58).

To date, they have mostly been exploited in the pre-clinical setting. The disease models on which they were tested include AD, amyotrophic lateral sclerosis (59), melanomas (60), chemotherapy-

induced enteropathy (61) and reperfusion-induced damage models (62). In the clinical setting, a radiolabeled version of ML-10 has been used to monitor the response to radiotherapy of brain metastases (63).

These molecules are able to cross the blood-brain barrier, and are therefore theoretically suitable for use in neurodegenerative conditions such as AD, PD, and glaucoma; however, they have never been tested on humans for this purpose. Moreover, a high dose is required to reach desirable image performance in most cases, therefore raising concern regarding the possible toxic events related to their use.

Caspase activation detection

FLIVO™

Caspases are a family of endoproteases that have a fundamental role in apoptotic and inflammatory processes. In apoptosis, caspase cascade activation occurs through extrinsic or intrinsic signals. The extrinsic pathway is triggered by ligands binding to extracellular death receptors, whilst the intrinsic pathway responds to intracellular stress signals such as hypoxia, DNA damage, reactive oxygen species, misfolded protein accumulation and mitochondrial damage. Irrespective of the trigger, the cascade begins with activation of 'initiator' caspases that, once active, are able to cleave and switch on 'executioner' caspases. Executioner caspases are responsible for DNA fragmentation which eventually leads to cell death.

FLIVO (FLuorescence in vIVO) is a family of fluorescent caspase inhibitors that allow visualisation of in-vivo and in-vitro apoptosis. These tracers can be directly injected into the circulation and selectively accumulate in apoptotic cells. Being able to also cross the blood-brain-barrier, they have potential in the study of brain and ocular neurodegenerative conditions by selectively highlighting cells cells undergoing caspase-dependent apoptosis, the majority of programmed cell death (64).

FLIVO technology has been used only in the pre-clinical setting for in vitro and in vivo studies. It has been exploited in oncology to develop new chemotherapeutic agents and cancer vaccines. In ophthalmology, FLIVO has been used to monitor activity of diabetic retinopathy (65), glaucoma (66,67), retinitis pigmentosa(68), blue-light induced retinal damage and age-related macular degeneration (69).

Z-DEVD-aminoluciferin

Luciferins are a family of bioluminescent molecules (usually proteins) found naturally in animals such as fireflies. Luciferins are activated via enzymatic cleavage by luciferases. Activated luciferins are able to release energy through light emission. Z-DEVD aminoluciferine is a modified luciferin. As a caspase 3/7 substrate, it is not activated by luciferases but rather by these specific caspases, and therefore has been used to monitor the activity of these caspases in vitro (70). Upon cleavage by the aforementioned enzymes, the subsequent bioluminescence is exploited as marker of apoptosis. In vivo apoptosis detection with an injection of Z-DEVD aminoluciferin has been shown in mice models of tumour xenografts (71). This tool has also been used in the drug development setting, however, its role in the clinical field has not been explored yet.

Diagnosis

The current clinical gold-standard for glaucoma diagnosis is standard automated perimetry (SAP). However, this method possesses practical and analytical disadvantages, in that it requires a long time to conduct, requires serial measures through over several years to detect change, is subject to a learning process that is variable according to the patient's fitness, concentration and comprehension (72,73). Moreover, some SITAs protocols, such as the 24-2, have been advocated as inadequate for early disease diagnosis (74). In the time it takes for a patient to develop a visual field defect detectable by current methods, approximately 30% of RGCs are lost. The time span of this pre-clinical phase has been estimated to range from 2 to 8 years, according to the progression rate (6). In this setting, structural OCT imaging has been shown to be the most promising detector of pre-perimetric RGC loss. This is not only in glaucoma, but also in other neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease and optic neuritis (1). However, the considerable variation between individuals limits the diagnostic value of single measures (75,76).

Raised IOP has been shown to be associated with progression of visual field defects (10,76). Therefore, the majority of medical and surgical treatments for glaucoma target intraocular pressure (IOP), with the aim of reducing it; however, it is an imperfect surrogate due to the wide interindividual variability of its pathologic effects (77).

In contrast to the biomarkers discussed above, DARC has the potential to minimize the number of years required to make a diagnosis by offering an indication of the severity of disease on first visit, potentially prior to the formation of significant visual field defects. The imaging of an active process implies prognostic value, and is arguably an important drawback of OCT and perimetry, whereby any

defects seen are not necessarily progressive (Figure 1). It is still unknown if prognosis can also be determined in other neurodegenerative conditions. Additionally, DARC is practical to implement, and not so reliant on the patient's ability to conduct the test. The inter-individual variability and change in disease states that will determine diagnostic ability, and the repeatability of the test that will determine the potential in clinical trials are to be determined in future studies and publications.

Conclusions

Several ocular and extraocular neurodegenerative diseases share the common feature of early and pathological death of retinal cells. This provides a potential early diagnosis window in which to delay and possibly halt pathologic processes before they cause significant harm. Apoptosis detection in retinal cells seems a plausible means to achieve this goal, with different strategies and technologies in the pipeline, with DARC already proven safe in humans. Their transition from bench to bedside may in the near future aid diagnosis, prognosis, follow up, therapeutic tailoring and drug development in the field of ophthalmology and neurology.

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Cohen SR, Gardner TW. Diabetic retinopathy and diabetic macular edema. In: Nguyen QD, Rodrigues EB, Farah ME, Mieler WF, Do DV, editors. Retinal pharmacotherapeutics. Dev Ophthalmol. Basel: Karger; 2016. Vol. 55; p. 137–46.

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Figure Legends

Fig. 1. Legend text.

Fig. 2. Legend text.

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