Detecting Apoptosis as a Clinical Endpoint for Proof of a 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 visualising 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 summarise the transitioning of techniques detecting apoptosis from bench to bedside, along with the future possibilities they encase.

Keywords

Apoptosis · Imaging · Retina · Neurodegeneration · Glaucoma

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 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.

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 10 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 fibres 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 localised 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 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 250 µm [18]. Larger drusen have a tendency to fuse leading to pigment epithelial detachment. 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 dUTP nick-end labeling) [20].

DR is a common complication of diabetes mellitus. It is the most common cause of vision loss among 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 cell 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% 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
Apoptosis detection in vivo exploits modifications of apoptotic cells undergoing programmed cell death: PS exposure, changes in apoptotic membrane imprint and caspase activation.

Since its first description in 2004, Detection of Apoptosing Retinal Cells (DARC) technology has been used for the assessment and follow-up of different animal models of degenerative retinal diseases for natural history characterisation and the study of novel neuroprotective agents [32]. 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 [33]. 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” [34]. PS represents an “eat me” signal for phagocytes, removing apoptotic debris to prevent pro-inflammatory consequences [35].

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 and single-photon emission computed tomography nuclear imaging techniques [36]. The main applications of these types of studies related to oncology, inflammatory bowel diseases, myocardial infarction and strokes [37–39]. For DARC to accomplish retinal imaging, annexin A5 has been fluorescently labelled using both 488-nm and near-infrared 776-nm tags with excitation and emission spectra of 495–519 and 771–793 nm, respectively (ANX776) [40].

Modified confocal scanning laser ophthalmoscopy (cSLO) is used to image the retina using the in-built fluorescent detection systems, providing high-contrast retinal images [41]. The latter near-infrared wavelength of ANX776 is aligned to that of indocyanine green for which imaging set-ups are in widespread use by medical retina specialists. The field of image acquisition is between 30 and 55° and can be either centred on the fovea or the optic disc. Compensation for non-enhancing structures and non-linear distortion is performed post-acquisition processing [42], with quantification of apoptosing cells performed via a template-matching approach to count hyperfluorescent spots, a count known as the “DARC count” [43].

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 [32]. DARC has also been used to characterise the rela-
tionship between raised IOP and retinal apoptosis, an ini-
tiating injury used in many animal models of glaucoma
[44]. Following on from this work, a novel staurosporine-
induced rat ocular hypertension model was demonstrat-
ed and used to investigate the neuroprotective effect of
modulating glutamate-induced excitotoxicity [45].
DARC was used to investigate the role of amyloid plaques
in retinal tissue and their relationship with retinal apop-
tosis. 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 [46].

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 ex-
amination [47].

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 [48]. This last study prompted further in-
vestigation 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 [49]. 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 apop-
tosis in vivo [50]. Brimonidine, an alpha-2 adrenergic re-
ceptor agonist, was shown to be able to reduce the rate of
retinal cell apoptosis through an IOP-independent mech-
anism related to amyloid precursor protein aggregation
modulation [51]. Coenzyme Q10 was also shown to fa-
cilitate significant reduction of retinal apoptosis in vivo
using DARC [52]. A liposomal formulation of rosigli-
tazone, a peroxisome proliferator-activated receptor-
gamma agonist, was used with DARC in a rotenone-in-
duced rat model of PD [53]. The results of the study
showed protection both at the level of the retina with re-
duced retinal apoptosis, and in the nigrostriatal pathways
of the brain [53]. 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 neuropro-
tective abilities in vivo [54, 55]. DARC demonstrated
both were able to provide significant reduction in retinal
apoptosis in rat models of ocular hypertension [54, 55]. 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 re-
growth and preventing secondary RGC neurodegenera-
tion using DARC [56]. Eventually, DARC technology has
been used in a recent study to monitor the effects of top-
ical recombinant human nerve growth factor (rh-NGF)
in a rat model of glaucoma. The results showed that par-
tial optic nerve transected rats receiving topical rh-NGF
had a non-statistically significant difference in RGC
apoptosis rate if compared with naive controls, both at
DARC count and histologic sample examination [57].

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 [40]. This was a sin-
gle-centre, open-label, proof-of-concept clinical trial de-
digned to primarily assess safety, and secondarily efficacy
of DARC imaging in humans [40]. The study was carried
out on 8 healthy volunteers and 8 patients affected by pro-
gressing glaucoma. These subjects were randomly allocat-
ed to one of the different ANX776 dosage groups. Each of
the four dosage groups included 2 glaucoma patients and
2 healthy controls. After the single intravenous injection
of ANX776, retinal imaging was performed to visualise fluo-
rescently labelled retinal cells at 15, 30, 60, 120, 240, and
360 min (Fig. 1). 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 [43, 58].

All subjects were required to attend three visits, and a
follow-up at 30 days. They underwent standard eye ex-
amination, including best-corrected visual acuity, to-
ometry, gonioscopy, dilated fundus examination, optical
coherence tomography (OCT) and standard automat-
ed 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 po-
tential of DARC as a predictive surrogate marker.
Apoptosis Detection in Retinal Diseases

No patients were withdrawn from the study, and no serious adverse events occurred. The study reported only isolated cases of discomfort during phlebotomy, haematoma 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 min, with maximal concentration proportional to the dose administered. These results were consistent with other studies using radiolabelled annexin A5 [59].

The greatest difference between healthy controls and glaucoma patients was seen when ANX776 was administered at a dose of 0.4 mg (\(p < 0.01\)). Multivariate analysis showed a 2.4-fold higher DARC count in glaucoma patients across the 6 h monitored (95% CI 1.4–4.03; \(p = 0.003\)). The 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 a pathology similar to AD [60]). The patients enrolled for this study received a single shot of ANX776 at a dose of 0.4 mg and were then imaged at 15 min, 2 and 4 h 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**

PSVue 550 (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 [61], with no direct retinal toxicity of the probe [61].

In the experiment conducted by Mazzoni et al. [61] eye penetration of PSVue 550 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 of retinal degeneration the dye was able to reach the posterior segment of the eye. However, only apoptotic photoreceptors of RCS rats could be visualised. 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 PSVue 550 or pSIVA; however, only PSVue 550 was able to reach the posterior retina upon topical application. These results were obtained upon histologic sample examination fluorescence microscopy [61].

PSVue 550 toxicity was tested through photoscopic full-field electroretinograms on dark-adapted rats at 3 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 [61].

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 h after topical subministration. Moreover, after 72 h, there was no statistical dif-
ference in the level of fluorescence of treated and control eyes. Therefore, the transitory nature of PS Vue 550 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 a light-induced retinal damage model [61]. 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 [61]. 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 300–700 Da. These selectively cross the apoptotic plasma membrane and accumulate in the cytoplasm of dying cells [62]. 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 recognised 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′-dansyl-L-cystine, 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 [62].

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 [63], melanomas [64], chemotherapy-induced enteropathy [65] and reperfusion-induced damage models [66]. In the clinical setting, a radiolabelled version of ML-10 has been used to monitor the response to radiotherapy of brain metastases [67].

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 undergoing caspase-dependent apoptosis, the majority of programmed cell death [68].

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 the activity of DR [69], glaucoma [70, 71], retinitis pigmentosa [72], blue-light induced retinal damage and AMD [73].

**Z-DEVD-Aminoluciferin**

Luciferins are a family of bioluminescent molecules 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-aminoluciferin 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 [74]. Upon cleavage by the aforementioned enzymes, the subsequent bioluminescence is exploited as a marker of

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Apoptosis. In vivo apoptosis detection with an injection of Z-DEVD-aminoluciferin has been shown in mice models of tumour xenografts [75]. 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. However, this method possesses practical and analytical disadvantages in that it requires a long time to conduct, requires serial measures over several years to detect change, is subject to a learning process that is variable according to the patient’s fitness, concentration and comprehension [76, 77]. Moreover, some SITA protocols, such as the 24-2, have been advocated as inadequate for early disease diagnosis [78]. 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 timespan 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 AD, PD and optic neuritis [1]. However, the considerable variation between individuals limits the diagnostic value of single measures [79, 80].

Raised IOP has been shown to be associated with progression of visual field defects [10, 80]. Therefore, the majority of medical and surgical treatments for glaucoma target IOP, with the aim of reducing it; however, it is an imperfect surrogate due to the wide interindividual variability of its pathologic effects [81].

In contrast to the biomarkers discussed above, DARC has the potential to minimise 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 (Fig. 2). 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.

**Conclusion**

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.
Conflict of Interest Statement

M.F.C. is a named co-inventor on granted patent EP 2231199B1 and published patent WO 2011055121 A1 owned by UCL and related to DARC technology. The other authors declare no conflicts of interests.

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References

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