

Chapter 2

Detection of Apoptosis: From Bench Side to Clinical Practice

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Abstract

Apoptosis or programmed cell death is implicated in several pathological conditions, such as cancer and neurodegenerative diseases. An increasing number of therapies are developed by targeting apoptosis signaling components to either induce or inhibit apoptosis in target cells. For these reasons, it is critical to develop appropriate analytical methods for the detection of apoptotic cell death in the context of monitoring relevant disease progression and therapeutic effects of clinical treatments (e.g., chemotherapy in cancer patients). This review provides an overview of the currently used methods for detection of apoptosis and their applications in research and clinical practice.

Key words Apoptosis, Apoptosis detection, In vitro apoptosis detection, In vivo apoptosis detection, Clinical apoptosis detection, DNA fragmentation, TUNEL, Caspase activation detection, Phosphatidylserine externalization

1 Introduction

Apoptosis or programmed cell death is a highly organized cellular process for removing unwanted cells from the body during organ development, tissue remodeling, and immune responses. Apoptosis is thought to be physiologically advantageous because early apoptotic cells are cleared by phagocytosis before they lose their plasma membrane permeability barrier [1–3]. In this manner, apoptotic cells are degraded within the macrophages. Loss of control of programmed cell death (resulting in excessive apoptosis) can lead to neurodegenerative diseases, hematologic diseases, and tissue damage. For example, the progression of HIV is directly linked to excessive, unregulated apoptosis. On the other hand, insufficient or defective apoptosis is linked to the development of cancer progression and drug resistance to chemotherapy. As such, an increasing number of drugs have been approved or are under development which target specific aberrant signaling components of cell death or survival pathways [4–6], including small molecule inhibitors and

therapeutic proteins [7]. Additionally, while the primary goal of chemotherapy is to kill cancer cells by any means, a secondary goal is to have those cells die by apoptosis so that they may be cleared quickly and “quietly” by neighboring phagocytic cells. For these reasons, it is critical to develop appropriate analytical methods for the detection of apoptotic cell death for monitoring disease progression and for the effects of therapeutic intervention like chemotherapy.

Apoptosis is characterized by a discrete set of biochemical steps and morphological changes that include the activation of caspases, translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane, chromatin condensation, and fragmentation of the cell into subcellular parts called apoptotic bodies [8]. There are two major apoptosis pathways that can lead to caspase activation: the mitochondria-directed intrinsic pathway and the death-receptor mediated extrinsic pathway [9–11]. The intrinsic apoptotic signaling pathway is triggered in response to various stress signals including DNA damage, γ -irradiation, hypoxia, and survival factor deprivation. Intrinsic apoptosis involves the release of mitochondrial factors (e.g., cytochrome c) that signal downstream programmed cell death events. By contrast, the extrinsic apoptosis signaling pathway is mediated through the death receptors expressed on the cell surface membrane. These receptors, including TNF receptor 1 (TNFR1), Fas, DR4, and DR5, are characterized by an intracellular death domain that can be selectively activated by their cognate ligands such as TNF, Fas ligand (FasL), and TNF-related apoptosis inducing ligand (TRAIL) [12–15]. Both intrinsic and extrinsic apoptosis pathways lead to activation of a cascade of cysteine-dependent aspartyl proteases, known as caspases, which catalyze the cleavage of cellular substrates at specific amino acid sequences (e.g., DXXD for caspase 3) [16]. Another distinct feature of apoptosis is the translocation of phosphatidylserine from the inner leaflet to the outer layer of the plasma membrane [17].

2 In Vitro Apoptosis Detection Methods

Because Apoptosis entails a vast number of sequential biochemical events, thus providing many check points at which detection of apoptosis can be accomplished. This review focuses on apoptosis detection methods that are designed by exploiting detection of the flipping of phosphatidylserine to the extracellular side of the plasma membrane, mitochondrial membrane permeabilization, DNA fragmentation, and intracellular caspase activation (Fig. 1).

Fig. 1 (continued) mitochondrial intermembrane proteins (e.g., cytochrome c) through ELISA, confocal microscopy, or high performance liquid chromatography techniques. DNA fragmentation is measured either through gel electrophoresis or end point TUNEL immunohistochemical visualization.

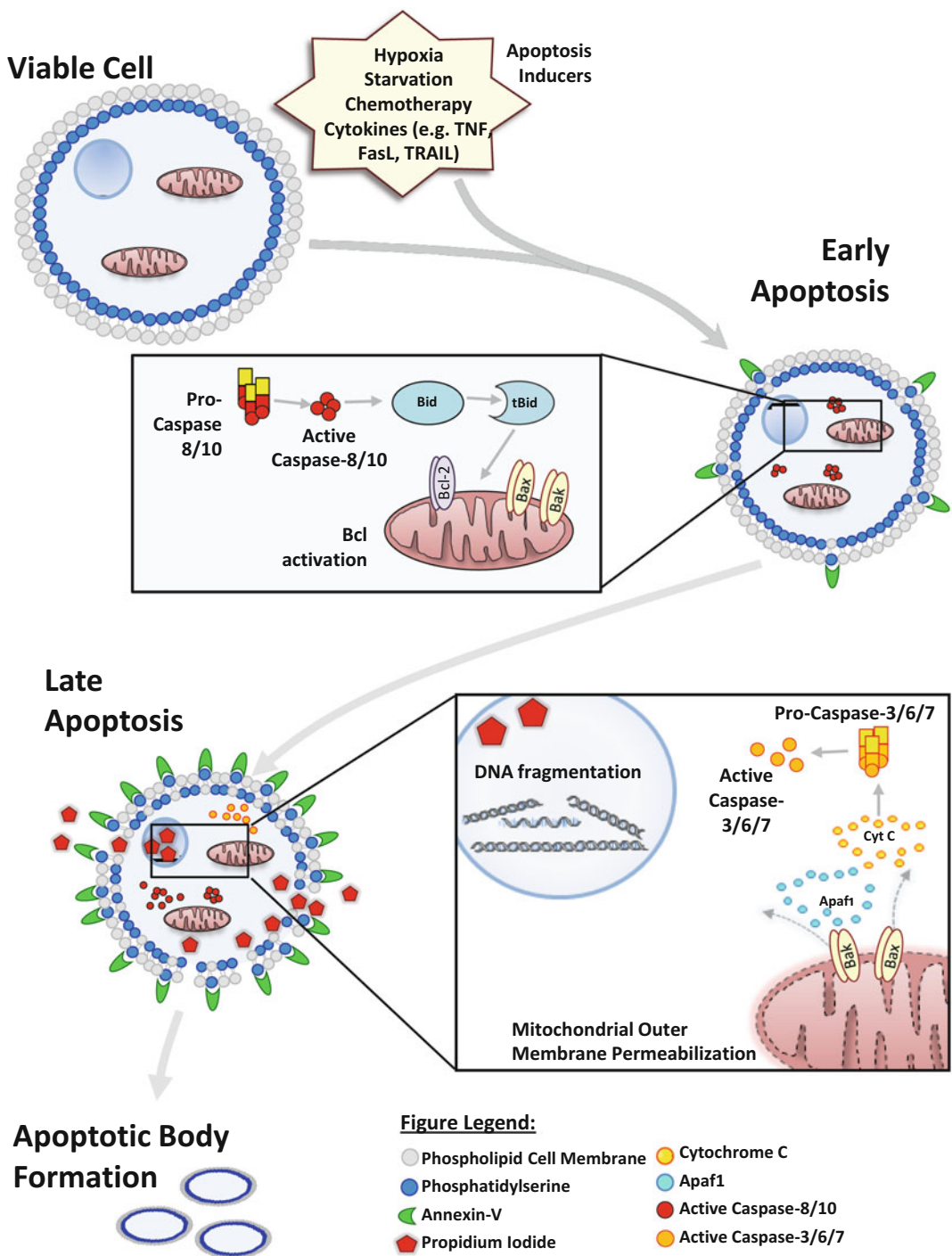


Fig. 1 An illustration of important apoptotic biochemical events that are often exploited to detect induction of apoptosis. Apoptosis is induced through varying endogenous and exogenous mechanisms such as hypoxia, starvation, chemotherapy, and targeted cytokines; and can be detected by exploiting one of the many biochemical events that occur during the programmed cell death. Loss of membrane symmetry and integrity can be visualized using labeled Annexin-V which binds to externalized phosphatidylserine on the cell membrane and propidium iodide which binds DNA after outer membrane permeabilization during late stages of apoptosis. Activated caspases can be detected and quantified using caspase labeling probes and labeled caspase substrates; while mitochondrial membrane permeabilization can be detected by identification of the release of

2.1 Phosphatidylserine Extracellular Flipping

Phosphatidylserine (PS) normally faces the inside leaflet of the cell membrane; however, during the onset of apoptosis PS flips and is exposed extracellularly. Flow cytometry has been widely used to detect cell populations with exposed PS. This detection is accomplished by exploiting the tight binding that exists between the anti-coagulant protein Annexin-V and PS. In these experiments, Annexin-V is conjugated to various fluorophores [18, 19] allowing for efficient labeling of apoptotic cells. Alternatively, detection approaches utilizing Annexin-V labeled with quantum dots (QDs) have advantages in robustness and sensitivity due to their semiconductor properties [20, 21]. PS extracellular flipping is one of the earliest processes that occurs during apoptosis, and is therefore used to detect cells undergoing early stage apoptosis. PS/Annexin-V labeling is often complemented with DNA binding dyes such as 7-amino-actinomycin D (7-AAD) or propidium iodide (PI) which can only penetrate the cell membrane during late stages of apoptosis and necrosis [22]. Together this approach allows for differentiation of healthy cells from cells undergoing early or late stage apoptosis and also necrosis.

2.2 Mitochondrial Membrane Permeabilization

Mitochondrial membrane permeabilization is another cellular change often used to detect apoptosis induction. Physical alterations in mitochondrial structure during apoptosis have been visualized by electron microscopy [23, 24]. However, this approach is limited regarding automation and quantification. Alternatively, analysis of the cellular redistribution of proteins that commonly reside in the intracellular space of the mitochondria is a technique often performed to assay mitochondria permeabilization, and therefore apoptosis [25]. This has traditionally been accomplished by immunoblot and immunofluorescence detection of released mitochondrial intermembrane spaced proteins, such as apoptosis-inducing factor (AIF) and cytochrome c (cyt c) [26, 27]. To aid in assay throughput, ELISA-based immunoassays have been developed for the detection of released cytochrome c [28]. Interestingly, confocal microscopy of GFP-tagged cytochrome c revealed important kinetic information, and has indicated that cytochrome c release can precede PS extracellular flipping and loss of plasma membrane integrity [29]. Similarly, high performance liquid chromatography (HPLC) has been used to detect mitochondrial metabolites that have diffused due to membrane permeabilization [30]. Alternatively, other assays have been developed that monitor the activity of the mitochondrial respiratory chain. These assays classically involve the conversion of tetrazolium salts into colored products; only occurring in the presence functional mitochondria. This technique is routinely used as a measure of cell viability [31–33]. However, lengthy incubation times and the inability to differentiate between growth arrest and true cell death are major limitations of the mitochondrial respiratory chain assays. Lastly,

many cationic fluorophores have been exploited in order to detect changes in mitochondrial transmembrane potential generated during apoptosis [34, 35].

2.3 DNA Fragmentation

DNA fragmentation is yet another key feature of apoptosis. DNA fragmentation is initiated by activated endonucleases, and sequentially yields high molecular weight DNA fragments that are further cleaved into oligonucleosomal fragments of 180–200 base pairs [36–38]. Apoptosis has historically been detected through visualization of these DNA fragmentation patterns. Specifically, conventional gel electrophoresis has been utilized in separating and visualizing low molecular weight DNA patterns, and has defined the characteristic “laddering” pattern as a hallmark of apoptosis [38, 39]. Additionally, pulse field and field-inversion gel electrophoresis have been used to resolve the larger high molecular weight DNA fragments [40, 41]. Due to its simplicity and sensitivity, single cell gel electrophoresis has been used to detect changes in DNA degradation, and therefore apoptosis at the single cell level [42]. This approach is advantageous in its ability to identify specific types of DNA damage such as single and double strand breaks. Collectively, gel electrophoresis techniques have limitations in assay time, automation, and quantification. Fragmented DNA can also commonly be detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) [43, 44]. In TUNEL, terminal deoxynucleotidyl transferase recognizes the 3′-OH termini of DNA breaks caused by induction of apoptosis. The enzyme then catalyzes the addition of labeled dUTPs into the damaged DNA. This labeled DNA can be visualized either by fluorescence microscopy or cytometry [45]. One major advantage of this approach is when complemented with PI staining, the phase of the cell cycle where apoptosis is occurring in can be determined [46]. However, cell fixation requirements and lengthy staining protocols are experimental limitations.

2.4 Caspase Activation

Caspases play an essential role in the execution of apoptosis, and their activation is often used as a marker of apoptosis. The availability of high quality antibodies specific to pro-caspases and their cleaved active forms offers a convenient tool for monitoring apoptosis under both in vitro and in vivo settings. Although caspase activation can be easily detected by immunoblot analysis, this approach has several limitations. In efforts to aid in assay robustness, sensitivity, repeatability, and automation there has been a strong push for the development of assays that can detect (1) labeling of active caspases and (2) cleavage of caspase substrates. In regard to caspase labeling, apoptosis detection through the use of fluorochrome-labeled inhibitors of caspases (FLICA) has been rigorously demonstrated [47–49]. FLICA-based probes are cell membrane permeant fluorescent ligands that covalently bind to the

caspase active site. Cells labeled with FLICAs can be detected using fluorescence microscopy, flow cytometry, or laser scanning cytometry (LSC). Recently, detection of caspase activation with FLICA has been accomplished in living lamprey brains and spinal axons, indicating the utility of this approach in an *in vivo* setting (*vide infra*) [50, 51]. However, a drawback of the FLICA approach is that in some instances these probes have actually been found to protect cells and prevent apoptosis [52, 53], complicating apoptosis detection.

Because caspases recognize and cleave specific amino acid sequences in substrate proteins, consensus peptides can serve as excellent biosensors for apoptosis detection [16]. Specifically, fluorophores such as 7-amino-4-methylcoumarin (AMC), 7-amido-4-trifluoromethylcoumarin (AFC), or rhodamine 110 have been covalently conjugated to caspase substrate peptides [54]. When covalently linked to a peptide, total fluorescence is quenched. However, in the presence of active caspase, the substrate is cleaved and the fluorophore is liberated from full length peptide yielding a pronounced fluorescence signal. In this design, high fluorescence backgrounds can often be one major obstacle. This has led to the evolution of fluorescence resonance energy transfer (FRET) as a powerful technique in detection of active caspase [55, 56]; where fluorescence donors and quenchers have been covalently attached to the termini of caspase substrate peptides. The intact substrate orients the fluorescence donor and acceptor in close proximity allowing for efficient FRET. Upon induction of apoptosis and caspase activation, the caspase substrate peptides are cleaved, allowing a distance dependent decrease in FRET. In many instances, the caspase biosensor is generated separately before addition to cells. This creates a major obstacle in cell permeability and can lead to a requirement of cell lysis, which adds undesirable complexity.

With the advancement of recombinant DNA technology, fluorescent fusion proteins have been engineered to act as excellent caspase/apoptosis FRET biosensors. In the design, one fluorescent protein acts as a donor while the other functions as an acceptor. Each protein is linked by a caspase specific peptide linker. As a cellular biosensor, this approach eliminates the need for additional substrate processing steps, such as cell penetration. Using this method, changes in FRET have been determined using many different fluorescent protein pairs [57–59]. Most commonly, FRET based detection of caspase activation relies on either microscopy or flow cytometry [58, 60–63]. While providing valuable information, this operating procedure is not ideal for high throughput formats. Notably, there are sparse examples reported which utilize a throughput plate format [64, 65].

Recently our lab has generated a novel cell-based FRET biosensor that allows for an automated detection of apoptosis induced by anticancer drugs [66]. Specifically, MDA-MB-231 breast cancer

cells have been engineered to stably express a CFP-linker-YFP fusion protein, wherein CFP functions as a donor and YFP as an acceptor for fluorescence resonance energy transfer (FRET). The linker contains both caspase 3 and caspase 8 recognition sequences, DEVD and IETD, respectively, allowing for sensitivity in drugs that function via either intrinsic or extrinsic apoptosis pathways. Upon caspase activation when cells are treated with a drug, the linker connecting CFP and YFP will be cleaved and subsequently will render a loss in cellular FRET signal. Importantly, the cell-based assay has been uniquely adapted to a microplate (e.g., 96-well plate) format.

Our platform has been found successful in quantifying the bio-activity of complex therapeutic proteins, such as TNF-related apoptosis-inducing ligand (TRAIL) and death receptor agonistic antibodies that work through extrinsic apoptosis pathways. Our assay was also successful in characterization of small molecule chemotherapeutic agents (e.g., camptothecin) that are effective by inducing intrinsic mediated apoptosis. Together these results showcase how our assay allows for an unprecedented detection of both caspase 3 and caspase 8 activity in a high throughput format. From a technical aspect, our assay is superior in sensitivity, simplicity, stability, convenience, and robustness when compared to the other described apoptosis assays. Also, our methodology has been uniquely adapted to eliminate background autofluorescence associated with cell culture media; this helps to achieve an improved signal-to-noise ratio and avoids high background subtractions which can complicate FRET calculations. Additionally, our platform has been adapted to a high-throughput screening format and was engineered to be easily setup in any lab. Most importantly, this assay closely reflects the mechanism of action of cancer drugs in killing cancer cells and can distinguish between drugs that solely trigger growth inhibition.

3 In Vivo Apoptosis Detection

Development of in vivo apoptosis detection techniques can be critical in assessing progression of several diseases and also in evaluating therapeutic effects of treatments, such as chemotherapy in cancer patients. Currently many in vivo apoptosis assays utilize noninvasive imaging modalities in order to detect tissues undergoing cellular apoptosis. These assays aid in individualized treatments for clinical efficacy, and allow for earlier predictions of the patient's response to therapy.

3.1 Changes in Diffusive Properties of Tissue and Tumor

Magnetic resonance imaging (MRI) has been used to monitor fluid diffusion gradients in tissues, and can distinguish tumors from healthy tissue due to high cellularity [67].

MRI is also often performed for the evaluation of tumor response to anticancer drugs; in these cases physical changes in tumor size and diffusion properties detected by MRI have been used to identify apoptotic cell death [68]. Specifically, diffusion weighted MRI (DW-MRI) has been used to measure increases in water diffusion for tumors in response to radiation, chemotherapies, and therapeutic proteins [69]. Tissues undergoing apoptosis will demonstrate an increase in apparent diffusion coefficient (ADC), used to represent water diffusion [70–73]. Another magnetic imaging modality, ^1H Magnetic resonance spectroscopy (MRS), has been used to quantify lipid concentration. Lipid concentration is known to increase in apoptotic cells [74] and this change in fat-water ratio is another established biomarker of tumor cell death [75, 76]. MRS has been found successful in detecting apoptotic cell death in cervical carcinoma [77] and in triple negative breast cancer treated with anti-DR5 antibody and carboplatin combination [78].

Although MRI approaches are commonly used in clinical practice, there has been a recent push towards the development of assays that can more directly and timely confirm the induction of apoptosis. This has been accomplished by labeling of apoptotic cells by exploiting the processes of PS extracellular flipping, changes in cell membrane imprint, DNA fragmentation, and caspase activation.

3.2 Phosphatidylserine Extracellular Flipping

An early indicator of apoptosis is the loss of membrane phospholipid asymmetry, causing PS to externalize on the plasma membrane [19, 79, 80]. One of the most common ways to detect this in vivo is by imaging for labeled Annexin-V. As mentioned earlier, Annexin-V has high affinity for PS. The in vivo uptake and biodistribution of Annexin-V has been investigated. Radiolabeled Annexin-V is imaged a minimum of an hour following intravenous injection, allowing for protein biodistribution [81–86]. The radiolabeled Annexin-V is cleared from circulation as quickly as three to seven minutes post injection, allowing for a clear indication of which tissues have taken up the protein [82]. Labeled Annexin-V can be monitored in 2D using scintigraphy or in 3D by positron emission tomography (PET) and single photon-emission tomography (SPECT). Tissue reconstructions are then created from rendered projections where Annexin-V is found to localize. Various radionuclides have been conjugated to Annexin-V, such as technetium-99m ($^{99\text{m}}\text{Tc}$) [83, 85, 87–89], Iodine-123 (^{123}I) [90], Iodine-124 (^{124}I) [91–94], Fluorine-18 (^{18}F) [95], and Copper-64 (^{64}Cu) [96]. Each differs in biological half-life, binding affinity, and kidney, liver, and spleen uptake. Radiolabeling and imaging of exogenously administered Annexin-V has been used to detect apoptosis following an acute stroke [81], multiple sclerosis [97],

allograft rejection [84, 86], arthritis [98], cardiac infarction [99, 100], cell death in patients with Alzheimer and dementia [101], and tumor response to anticancer therapies [84, 102]. Notably, in a Phase I/II clinical study, ^{99m}Tc -radiolabeled Annexin-V was successfully taken up in 7 of 15 patients that were presenting lung cancer, breast cancer, and lymphoma; and allowed for in vivo imaging of apoptosis in human cancers in response to chemotherapy treatment. Of these seven patients, four had a complete response and three had a partial response. Collectively, this work has strong implications for labeled Annexin-V functioning as a predictor of response to chemotherapy treatment.

Besides Annexin-V, synaptotagmin I [103–105] and the PS targeting monoclonal antibody fragment, PGN635 F(ab')₂ [94] have been used to bind PS and label apoptotic cells in vivo. Limitations of this methodology exist in pharmacokinetic problems associated with the large size of Annexin-V, nonspecific uptake, and its relatively slow clearance from the blood [85, 106, 107].

3.3 Changes in Apoptotic Membrane Imprint

Aposense molecules are low molecular weight compounds that have been developed to selectively invade, label, and image cells undergoing cell death. They target apoptotic cells by selectively binding to plasma membranes displaying changes in membrane potential, acidification of the outer leaflet and cytosol, and activation of the apoptotic scramblase system [108]. These probes then accumulate within the cytoplasm of apoptotic cells and allow for visualization due to their chemical properties. Interestingly, aposense uptake is caspase dependent [109] and has been shown to be selective for apoptotic cells, judged by TUNEL, hematoxylin and eosin (H and E) ex vivo staining, and Annexin-V staining [109, 110]. To date, several aposense molecules have been developed and reported: DDC [111], NST-732 [112], NST-729, ML-9 [113], and ML-10 [114]. Radiolabeled aposense compounds, such as ^{18}F ML-10, allow for positron emission tomography which adds tremendous value in clinical settings. Together this technology has been shown to be successful in identifying apoptosis induced by anticancer drugs, renal failure, neurodegenerative disease, and stroke [109–113]. Human studies revealed that ^{18}F ML-10 has advantages in biodistribution, stability, dosimetry, signal to noise, and safety [115]. It is currently being evaluated by clinical trials as a radiotracer for brain metastases, head and neck neoplasms, and non-small-lung carcinomas in response to radiation or chemoradiation (as provided by <https://clinicaltrials.gov>). Additionally, it has advantages in selectivity and can distinguish between cells dying through apoptotic and necrotic pathways [109, 114].

3.4 DNA Fragmentation

DNA fragmentation in biopsy samples is used to detect apoptosis induction through terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL). As previously mentioned, TUNEL staining identifies nicks within genomic DNA by detecting either fluorescence or DAB (3, 3'-diaminobenzidine) streptavidin-labeled dUTPs that are incorporated into damaged DNA by TdT. The degree of apoptosis is determined by the intensity of the TUNEL staining within apoptotic nuclei, as well as visualization of DNA fragments within the cytosol of the cell. Histology sections of biopsies are used to compare the baseline pretreatment and posttreatment samples to determine drug efficacy [116–118]. While this assay allows for direct apoptotic labeling within the tumor, it is an end-stage detection method and lacks the ability to monitor apoptosis in real-time to enhance personalized medicine. However, end point analysis of biopsy histology can be used in conjunction with imaging techniques to verify long-term responses.

3.5 Caspase Activation

Caspase activation is a well-known apoptotic biomarker that has been extensively showcased both in vitro and in vivo. Complementing detection of caspase activation with in vivo imaging provides early and selective detection capabilities for the onset of disease and therapeutic efficacy in relation to induction of apoptosis. As with traditional in vitro techniques, detection of caspase activation can be accomplished in vivo either by labeling active caspases with ligands or by detecting cleavage of caspase-based substrates.

Peptide-based caspase labeling ligands that mimic natural physiological substrates have been exploited for the in vivo detection of caspase activity, and therefore apoptosis. In one recent promising example, [^{18}F]4-fluorobenzylcarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone was developed and validated in vivo as a novel PET imaging probe capable of labeling active caspases and detecting apoptotic cell death triggered by anticancer treatment in mouse models of human colorectal cancer [119]. A major drawback of peptides based ligands is their overall modest uptake. In this regard, small molecule caspase ligands offer an attractive alternative. The most prominent examples exist within the isatin sulfonamide moiety family. Radiolabeled isatin sulfonamides, such as [^{18}F]WC-II-89 [120, 121], [^{11}C]4 (WC-98) [120], [^{18}F]ICMT-11 [122], and [^{18}F]WC-4-116 [123], are competitive caspase-3 inhibitors/labelers that can be traced using PET in order to detect caspase activation and apoptosis in vivo. However, a limitation exists in selectivity where other cysteine containing proteases can be labeled by these small molecule probes.

Caspase activity can also be measured by detection of enzymatic cleavage of substrate-based probes. In one recent example, a common caspase-3 peptide-based substrate (DEVD) was conjugated to a PET imaging radionuclide (^{18}F -CP18) on one side and

a cell permeating polyethylene glycol (PEG)-sequence on the other [124]. This allows the tracer to efficiently enter the cell and to be cleaved by activated caspases existing in apoptotic cells. The PEG sequence is subsequently released and causes the radionuclide labeled peptide to become trapped and accumulate within the dying cells. These tracers have advantages in selectivity and stability. Importantly, tumor bearing mouse studies revealed that in vivo PET signal images correlated well with caspase 3/7 activity and caspase-3 immunohistochemistry. This approach has significant advantages in target selectivity, signal to noise background, biodistribution, and clearance. However, chemotherapy induced apoptosis in mouse xenografts was not analyzed, limiting the knowledge of whether this probe can detect therapeutically relevant changes in apoptosis.

4 Conclusion

In summary, we outline and describe here many of the existing methods used to detect apoptosis both in vitro and in vivo. These detection methods are presented in order to show how in vitro method development has led to promising in vivo diagnostic tools. When attempting to select an appropriate apoptosis detection method, it is essential to carefully analyze the purpose and the end goal of the experiment. Our lab is primarily interested in the detection of apoptosis in the context of therapies that target apoptosis signaling components to either induce or inhibit apoptosis in target cells. In this regard, it is our opinion that selectivity and early detection is critical. Also, it is important to select a platform that can distinguish between growth arrest and true cell death. Therefore, we favor apoptosis detection methods that rely on caspase activation. For in vitro purposes, we feel that assays which are highly automated, sensitive, and quantitative stand out amongst other. Among the examples described, our cellular FRET-based detection of caspase activation in a 96-well plate format satisfies all of these criteria. For clinically relevant in vivo detection of apoptosis, it is necessary not only to correlate increases in apoptosis with the efficacy and toxicity of applied drugs, but also to track disease progression in real-time. Noninvasive imaging techniques that detect either PS binding to Annexin-V or caspase activation allow apoptosis monitoring that most closely resembles real-time. This is highly beneficial when observing patient response during drug treatment or in monitoring disease progression. Although further research and clinical studies are clearly needed, complementing PET-based imaging probes with detection of caspase activation seems highly promising in regard to selectivity, pharmacokinetic uptake, and clearance.

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