REVIEW

DOI: 10.37047/jos.2020-73477

Molecular and Biological Mechanisms of Apoptosis and its Detection Techniques

Suganya CHINNASAMY[a], Farhan ZAMEER[b], Krishnasamy MUTHUCHELIAN[a,b]

[a]Madurai Kamaraj University School of Energy, Environment and Natural Resources, Department of Bioenergy, Tamil Nadu, INDIA
[b]Dayananda Sagar University School of Basic and Applied Sciences, Department of Biological Sciences, Karnataka, INDIA

ABSTRACT Apoptosis (programmed cell death), a self-destructive cellular mechanism, is essential for various events like sculpting the body, responding to any abnormalities, and removal of unwanted/damaged cells. Either too little or a high level of apoptosis causes conditions, such as chronic neurodegenerative maladies including Alzheimer's and Parkinson's diseases and cancer, i.e., an uncontrolled cell development. A typical apoptotic process includes cell shrinkage, degradation of DNA and mitochondrial breakdown, formation of blebs, cell fragmentation, release of nucleotides and phosphatidylserine on the surface of the cell, evoking an "eat-me" sign to the phagocytes. The detection of cell death in cells and tissues has gained immense therapeutic potential. Although many key proteins of the cell cycle machinery and apoptotic signaling pathway have been identified, the molecular mechanisms of these proteins are still not clear. This review attempts to summarize the fundamental aspects and the molecular mechanism of apoptosis, recent advances in detection methodologies, as well as some of the negative aspects of the applied techniques.

Keywords: Apoptosis; programmed cell death; intrinsic/extrinsic pathway

Death is characterized as the state described by the end of indications of life and is caused by functional irregularity. Cell death is genetically controlled and can be categorized into programmed, such as apoptosis and autophagy, and unprogrammed, such as necrosis. Apoptosis is an active self-destruction process, which is influenced by a diversity of stimuli that induce highly similar structural changes. These morphological changes are chromatin build-up, cytoplasmic shrinkage, zeiosis and the arrangement of apoptotic bodies inside the nucleus. The concluding phase of apoptosis is characterized by cytoplasmic membrane fragmentation and phagocytosis of debris by macrophages or adjacent cells. Figure 1 illustrates typical changes in the cells undergoing apoptosis or necrosis and the possible differences are tabulated in Table 1. Death by apoptosis is differentiated from death by necrosis by the absence of inflammatory responses. Early in 1972, these observations were reported by Kerr et al. but were less significant in those days.[1] However, from embryogenesis to aging and from the normal tissue homeostasis to many diseases, apoptosis has been found to occur in various life stages and has become a field of importance in developmental biology, biogerontology, and cancer research. Malfunctioned apoptotic cascade may lead to several diseases like cancer due to its decreased rate and causes strokes and neurodegenerative disorders such as Alzheimer's, Huntington and Parkinson's diseases in an exaggerated state. Table 2 presents the timeline of cell death research.

HISTORY OF CELL DEATH RESEARCH (TABLE 2)

Correspondence: Krishnasamy MUTHUCHELIAN
Madurai Kamaraj University School of Energy, Environment and Natural Resources, Department of Bioenergy, Tamil Nadu, INDIA
E-mail: drmuthuchelian@gmail.com

Peer review under responsibility of Journal of Oncological Sciences.

Received: 22 Jun 2019 ***Received in revised form:*** 18 Nov 2019 ***Accepted:*** 21 Nov 2019 ***Available online:*** 14 Feb 2020

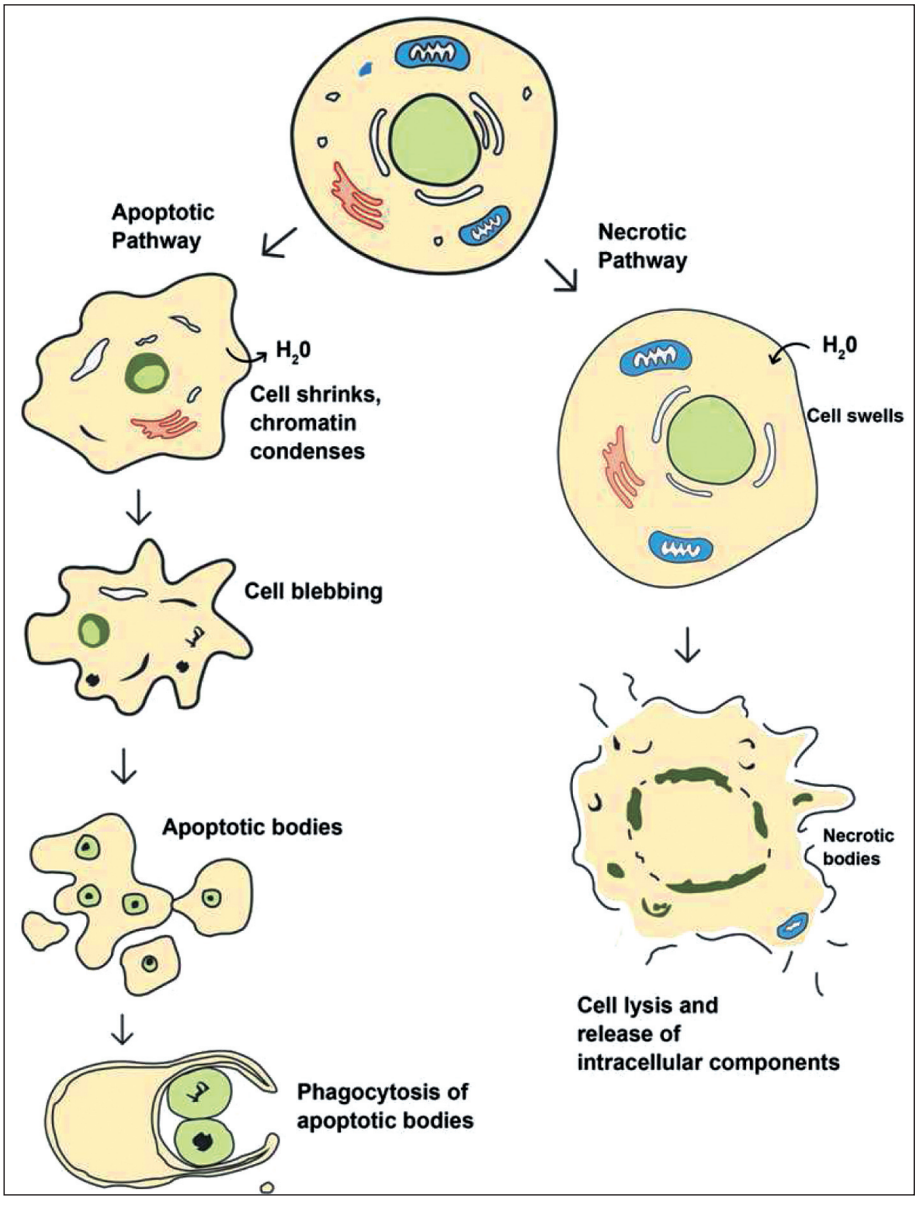

FIGURE 1: Structural changes of cells undergoing NECROSIS or APOPTOSIS.

WHY DO CELLS UNDERGO APOPTOSIS?

There are various reasons for a multicellular organism to carry out apoptosis.

FOR SCULPTING THE BODY

At specific stages of embryonic development, certain cells are programmed to die, to purge unwanted structures and sculpt the body to a precise form. In animals, apoptosis is required for various developmental processes, such as cartilage formation in vertebral animals, and metamorphosis in frog and insects. Further, unfertilized eggs after external fertilization also show signs of death through apoptosis. Plants also experience programmed cell death (PCD) for better determination of leaf and reproductive structures. Cell suicide during early developmental stages for the vertebrate limbs is depicted in Figure 2.

IN RESPONSE TO ABNORMALITY

Every cell has an exclusive mechanism to ascertain that the newly formed component is both morphologically and functionally ideal. If an imperfection is detected, the repair mechanism is initiated and if such

TABLE 1: Features of necrosis and apoptosis.

Features	Necrosis	Apoptosis
A. Morphological features		
Cell size	Increased	Decreased
Plasma Membrane Integrity	Disrupted	Intact
Nucleus	Caspase independent DNA fragmentation	Caspase dependent DNA Fragmentation
	Clumping and random degradation of nuclear DNA	DNA Laddering
		Aggregation of chromatin at the nuclear membrane
Cellular content	Begins with swelling of cytoplasm and mitochondria	Membrane blebbing and shrinking of cytoplasm
	Ends with total cell lysis	Fragmentation of cells
	No vesicle formation, complete lysis	Formation of Apoptotic bodies
	Disintegration of organelles	Leaky organelles
B. Biochemical features		
Energy requirement	Passive process	Active process
	ATP independent	ATP Dependent
	Occurs at 4°C	Does not occur at 4°C
Other Physiological Significant	Affects Neighboring cells	Does not affect neighboring cells
	Significant inflammatory responses	Lacks inflammatory responses
	Induced by non-physiological stimuli	Induced by physiological stimuli
	Macrophages involved in phagocytosis	Phagocytosis by both macrophages and adjacent cells

TABLE 2: Important events in the history of cell death.

Year	Event
4th century BC	Hippocrates: First used the term Apoptosis
1842	Vogt: Reabsorption of notochord during metamorphosis in amphibians
1872	Stieda: Death of chondrocytes in lacunae, cells found in cartilage connective tissue during ossification
1883	Metchnikoff: Activity of phagocytes
1890	Arnheim: Margination of chromatin
1955-1959	De Duve and co-workers spotted lysosomes and named them as “suicidal bags”
1965	The term Necrosis was coined
1973	Schweichel and Merker: categorized types of death
1976	Skalka et al.: DNA fragmentation is characteristic of apoptosis
1979	Farber and Fisher: Free radicals-root of cell suicide
1984	Wyllie et al.: Relate chromatin condensation to DNA fragmentation
1989-1992	Recognition of a cell surface receptor (Fas/APO-1/CD95)
1991-1994	p53-Identified as apoptotic inducer
1992	Phosphatidylserine is a marker of apoptosis
1993	Apoptosis toward cancer cell was shown to be induced by GSA and WGA
1996	Caspases defined
1996-1997	Significance of the liberation of cytochrome c for inducing apoptosis
1997	Phase I Clinical trial to Bcl-2 using antisense RNA
1999	Development of M30 Antibody for the detection of early apoptosis
1997	Phase I Clinical trial to Bcl 2 using antisense RNA
1999	Development of M30 Antibody for the detection of early apoptosis
2002	Genes that control apoptosis was identified by studying C. elegans
2003	Horvitz: Genetic details of apoptosis
2004	Formigli et al.: Balance between cell division and cell death-essential for tissue maintenance and organ homeostasis
2005	Degterev et al.: Concept of Necroptosis

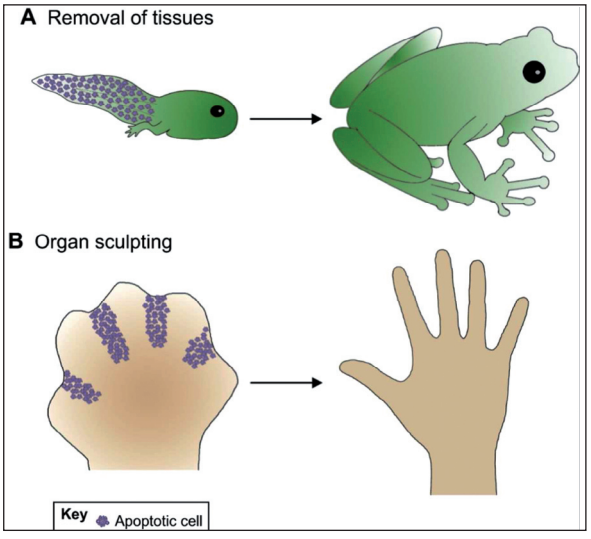

FIGURE 2: Cell suicide during early developmental stages.

repair machinery fails, the process of self-destruction is executed. Induction of cell suicide in the case of DNA damage is illustrated in Figure 3.

WHEN DO THE CELLS UNDERGO SENESCENCE?

Cells undergo apoptosis at the end of their life. This is achieved by a special structure called the telomere, which caps the end of each strand of DNA to protect chromosomes. As cells separate after some time, telomeres shorten and in the long run, cell division stops. A decrease in the length of telomeres is correlated with untimely cell aging.[2] As we age, telomeres are shortened, although they can likewise be shortened by stress, smoking, corpulence, absence of activity, and poor nutrition.[2,3] The immune system involves an arrangement of cells and molecules that safeguard the body from invaders, such as the pathogens and pathogenic determinants.[4] Cell suicide through apoptosis is the central procedure for dispensing with the trooper cells that act against the body itself.

BIOLOGICAL MECHANISMS OF APOPTOSIS

Apoptosis is an exceptionally unpredictable and advanced process including an energy-dependent cascade of molecular events.[5] There are two prime apoptotic pathways-the extrinsic/death receptor pathway and the intrinsic/mitochondrial pathway. These two pathways are connected and molecules in a single pathway can be manipulated by the other.[6] The extrinsic and intrinsic components converge at the same terminal, which is the execution pathway. This pathway is influenced by the cleavage of caspase-3 and mutations, degradation of cytoskeletal and nuclear proteins, formation of apoptotic bodies, cross-association of proteins, recognition of ligands by phagocytic cell receptors and finally engulfment by phagocytic cells. The granzyme A pathway triggers a parallel, caspase-free cell-signaling pathway.[7]

BIOCHEMICAL FEATURES

Apoptotic cells show a few biochemical adaptations, for example, protein cleavage, protein cross-connecting, DNA breakdown, and phagocytic recognition that together result in the distinct structural pathology described earlier.[8] Caspases are frequently expressed as inactive proenzymes in many cells and once active they trigger the protease pathway. Some procaspases can also be autoactivated. This proteolytic course, in which one caspase can enact different caspases, and sets a course of the response, opens up the apoptotic flagging pathway and along these lines prompts rapid cell death. Caspases have a proteolytic role and can cleave proteins at aspartic acid residues, though distinctive caspases have varying specificities that include identifying adjoining amino acids. When caspases are at first activated, there is an irreversible command toward cell death. At present,

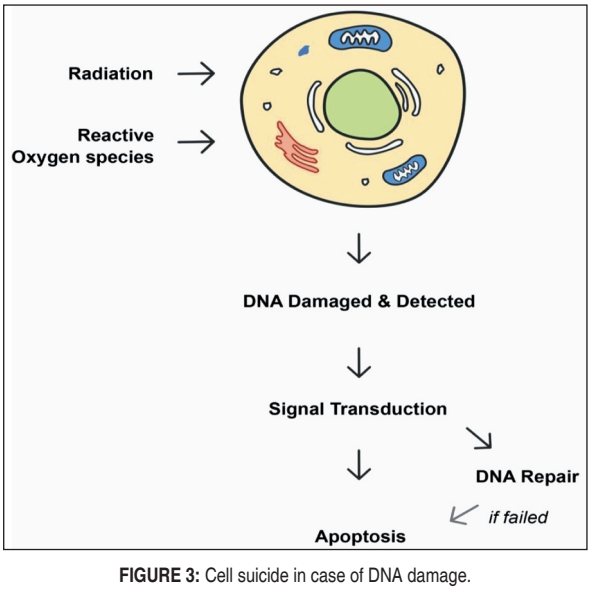

FIGURE 3: Cell suicide in case of DNA damage.

ten caspases have been distinguished and comprehensively classified into initiators (caspase-2, -8, -9, and -10), effectors or killers (caspase-3, -6, and -7), and provocative caspases (caspase-1, -4, -5).[9] Alternate caspases that have also been recognized identified include caspase-11, which manages apoptosis and cytokine development amid septic shock, caspase-12, which mediates apoptosis specific to the endoplasmic reticulum (ER) and cytotoxicity by amyloid-β, caspase-13, which is proposed to be a bovine gene, and caspase-14, which is exceedingly communicated in embryonic tissues and not in adult tissues.[10-14]

EXTRINSIC PATHWAY

The extrinsic pathway is initiated by extracellular signals. TNF-α (tumor necrosis factor), which is secreted by T-killer cell, induces apoptosis in targeted and suspected cells. TNF-α binds with its associated receptor and activates the death domain. TNF receptor-related death domain protein (TRADD) attaches to fas-associated death domain (FADD) and likewise, procaspase-8 gets associated with FADD. This complex sets the autocatalytic activity to begin the hydrolysis of the inhibiting segment initiating the caspase cascade and ultimately leading to the hydrolysis of proteins.

The extrinsic signaling pathway that begins apoptosis incorporates transmembrane receptor-intervened interactions. These incorporate death receptors that are members of the TNF receptor superfamily.[15] Individuals from the TNF receptor family share similar cysteine-rich extracellular regions and possess a cytoplasmic domain of nearly 80 amino acids called the "death domain".[16] This death domain plays a critical role in the intracellular signaling pathways. The best-characterized ligands and corresponding death receptors include FasL/FasR, TNF-*α*/TNFR1, Apo3L/DR3, Apo2L/DR4, and Apo2L/DR5.[16-20] The sequences of events that characterize the extrinsic phase of apoptosis are best described by the FasL/FasR and TNF-*α*/TNFR1 models. In these models, a cluster of receptors appears and its aggregation with homologous trimeric ligand occurs. Upon the binding of the ligand, cytoplasmic connector proteins are recruited, which display associated death domains that bind to the receptors.

The binding of Fas-ligand to Fas-receptor brings about the interlinking of the connector protein (adapter), Fas-associated protein with death domain (FADD). Likewise, the binding of TNF ligand to TNF receptor brings about the binding of the connector protein TRADD with the enrollment of FADD and receptor-interacting protein (RIP).[21-23] FADD associates with procaspase-8 to form a death-inducing signaling complex (DISC), which then results in the auto-catalytic activation of procaspase-8, which then actuates the execution period of apoptosis.[24] The binding of the c-FLIP protein to FADD and caspase-8 renders them ineffective and finally inhibits the death receptor-mediated apoptosis.[25,26] Another purpose of potential apoptosis regulation includes a protein called Toso, which has been shown to inhibit Fas-activated apoptosis in T cells by controlling caspase-8 processing.[27]

INTRINSIC PATHWAY

The intrinsic signaling pathway otherwise called the mitochondrial pathway is initiated by a wide array of non-receptor-mediated stimuli, which produce intracellular signals. These intracellular signals may act either positively or negatively. The positive trend includes activation of pathway *via* radiations, toxins, hypoxia, hyperthermia, viral infection, and free radicals. The negative interaction involves the absence or presence of a negligible amount of growth factors, hormones, and cytokines focused on the failure or the suppression of the cell-death programs, which trigger apoptosis. All these stimuli bring about changes in the internal mitochondrial membrane leading to the opening of mitochondrial transition core and loss of permeabilization, which activates the release of two principle clusters of pro-apoptotic proteins from the internal layer into the cytosol.[28] The first group comprises of cytochrome c and SMAC/DIABLO.[29-32] Cytochrome c binds to APAF-1 and procaspase-9 complex, forming apoptosome, which when activated, leads to the formation of caspase-9.[33,34] SMAC/DIABLO has been reported to promote apoptosis by inhibiting the activity of the inhibitors of apoptotic proteins (IAP).[35,36] Some mitochondrial

proteins have also been shown to interact with the IAP and suppress its action.[37] The second cluster of proapoptotic proteins, AIF, endonuclease G, and CAD are discharged from the mitochondria at the last stage after the cell has committed to die. Both AIF and endonuclease-G function in a caspase-independent manner. AIF translocates to the nucleus, where it causes fragmentation of the DNA into 50 to 300 Kb fragments and condenses the nuclear chromatin.[38] This initial form of nuclear condensation is termed as "stage-I" condensation.[39]

Endonuclease G translocates to the nuclease, carries out chromatin cleavage to deliver oligonucleosomal DNA fragments.[40] Likewise, caspase-activated DNase (CAD) translocates to the core, prompting oligonucleosomal DNA segments and advanced condensation of chromosomes, which is alluded to as "Stage-II". The different apoptotic mitochondrial events are entirely controlled and directed by the Bcl-2 group of proteins.[41] Though the precise machinery of the Bcl-2 family of proteins is not known, it is assumed that the tumor suppressor protein p53 has a unique role in the regulation of the Bcl-2 family of proteins.[42] Till date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-X, Bcl-XL, Bcl-XS, Bcl-W, BAG, and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. It is believed that the Bcl-2 family of proteins plays a crucial role in the release of cytochrome C from the mitochondria. The other two members of the Bcl-2 family, which support pro-apoptotic events, are PUMA and NOXA. PUMA plays a critical role in p53 mediated apoptosis, likewise, the raised articulation of PUMA is associated with the expanded articulation of Bax protein, prompting the basic changes and translocation into mitochondria. It lessens the mitochondrial membrane potential and culminates in the arrival of cytochrome C.[43] Like PUMA, NOXA is also an intermediate in p53-induced apoptosis. Studies have demonstrated that NOXA can translocate into the mitochondria and collaborate with the Bcl-2 group of anti-apoptotic proteins, appearing at the initiation of caspase-9.[44] In addition to the differential contribution of PUMA and NOXA, the dual pathway of p53 mediated apoptosis was also studied.[45] In ordinary cells, PUMA and NOXA intercede the calcium discharge pathway from the endoplasmic reticulum (ER). PUMA has been reported to interact with various anti-apoptotic Bcl-2 family members, but NOXA interacts particularly with Mcl-1 and A1 of this family.[46] Another oncoprotein, Myc has the potential to elicit apoptosis through p53-dependent and independent processes.[47] Figure 4 schematically represents the intrinsic and extrinsic pathways.

EXECUTION PATHWAY

The execution phase is considered to be the final stage of apoptosis, which causes the cells to shrink, form cytoplasmic blebs, and apoptotic bodies, and at the end, the neighboring parenchyma cells or macrophages phagocytose the apoptotic bodies. All the extrinsic, intrinsic, and perforin pathways end at a point initiating the executioner caspases. Caspases-3, -6, and -7 work as effectors or executioner caspases, and separate different substrates including PARP, cytokeratin, nuclear mitotic apparatus protein (NuMa), fodrin and α- tubulin proteins and bring about various other morphological and biochemical changes in cells undergoing apoptosis.[48] These changes lead to the release of cytochrome C and SMAC from the mitochondria followed by the formation of apoptosome and subsequent activation of caspases.

Cytochrome C coupled with APAF activates initiator caspases-8, -9, and -10. This, in turn, activates executioner caspases-3, -6, and -7. Of these, caspase-3 is believed to be the most fundamental of the executioner caspases, which especially acts as an endonuclease. CAD, which is complexed with its inhibitor, ICAD in cells, experiences apoptosis. The activated caspase-3 cleaves ICAD to release CAD. CAD at that point corrupts chromosomal DNA, condenses the chromatin, remodels the cytoskeleton, and makes the cells break down into apoptotic bodies. Phagocytosis of the apoptotic bodies is the last stage of apoptosis. Cells die because apoptosis attracts phagocytes by producing "eat-me" signals by releasing ligands for receptors present on phagocytic cells.

Apoptotic cells experience a variety of changes. A hallmark of the apoptotic stage is the externalization of the phosphatidylserine residue normally found

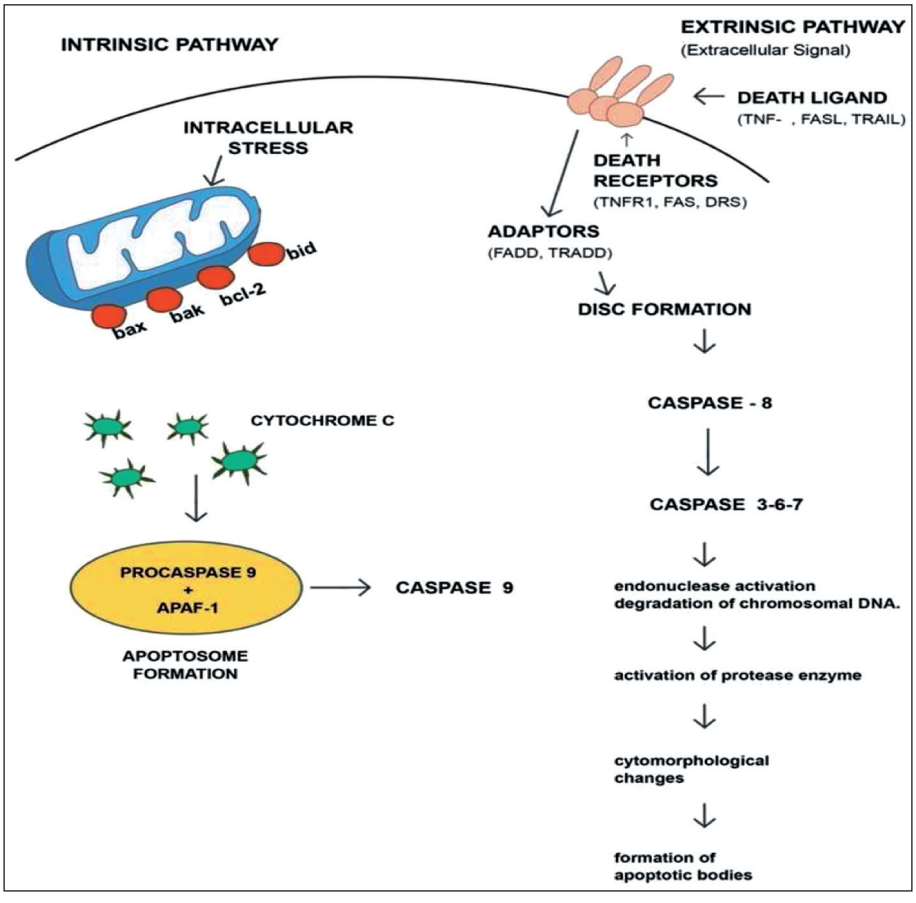

FIGURE 4: Schematic representation of apoptotoic events.

on the inner leaflet of the cell membrane, which is dependent on the process mediated by caspases.[49,50] The process of phagocytosis-like recognition, uptake, and degradation of apoptotic cells occurs very rapidly, preventing the occurrence of the inflammatory process. This is because the dying cells are removed before they lose their cell integrity, and no longer release their cellular content and are also the cause why the engulfment of apoptotic cells does not stimulate an inflammatory response. A few of the several receptors that are involved in the recognition and uptake of apoptotic cells are class A & B, CD68, the avß3 integrin, and phagocyte lectins. Some of these phagocytic receptors require a bridging molecule for the interaction to occur. ß2-Glycoprotein I and gas-6 are two important bridging candidates between apoptotic cells and phagocytic cells.[51] Table 3 lists the major proteins in the execution stage with common abbreviations and some of the alternate nomenclature used for each protein.

DETECTION OF APOPTOSIS

Apoptosis occurs through highly ordered sequences and to measure the activity, numerous conventional and novel techniques have emerged. Some of these methodologies are listed in the coming sections.

Every assay has its advantages and shortcomings. There are overlapping features between different forms of cell death. Hence, it is very important to carry out two or more assays to corroborate apoptotic cell death. Another important aspect is that certain assays are appropriate for cultured cells but cannot be used to investigate tissue sections. Therefore, care should be taken in choosing the appropriate method.

MEMBRANE INTEGRITY ASSAY

Dye Exclusion Test

This test determines the fraction of viable cells in suspension of dilute solutions of certain dyes such as try-

TABLE 3: Major proteins in the execution pathway.

Abbreviation	Protein name	Selected alternate nomenclature
Caspase-3	Cysteinyl aspartic acid-protease-3	CPP32, Yama, Apopain, SCA-1, LICE
Caspase-6	Cysteinyl aspartic acid-protease-6	Mch-2
Caspase-7	Cysteinyl aspartic acid-protease-7	Mch-3, ICE-LAP-3, CMH-1
Caspase-10	Cysteinyl aspartic acid-protease-10	Mch4, FLICE-2
PARP	Poly (ADP-ribose) polymerase	ADP ribosyltransferase, ADPRT1, PPOL
Alpha fodrin	Spectrin alpha chain	Alpha-II spectrin, fodrin alpha chain
NuMA	Nuclear mitotic apparatus protein	SP-H antigen
CAD	Caspase-activated DNAse	DNA fragmentation factor subunit beta, DFF-40, caspase-activated nuclease, CPAN
ICAD	Inhibitor of CAD	DNA fragmentation factor subunit alpha, DFF-45

pan blue (TB), eosin Y, nigrosin, alcian blue, safranin, erythrosine B (EB), propidium iodide, and 7-amino actinomycin D.[52-55] Cells that do not stain are determined to be alive, whereas cells that stain well thought to be dead. It is not normally an exact test since it shows just the structural integrity of the cell membrane.[54] In Figure 5, the staining of hepatocytes is shown. Although trypan blue, a diazo dye, exhibits the quality of a vital dye, a potential teratogenic effect has been reported.[56-60]

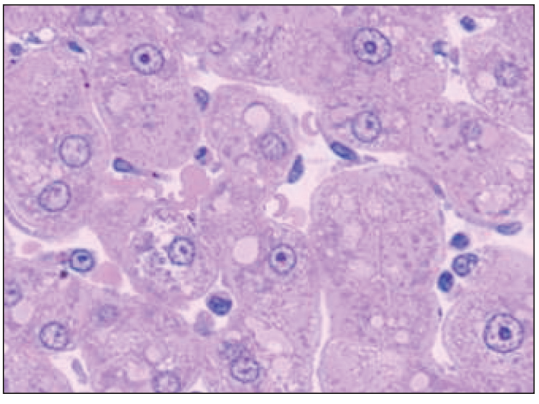

FIGURE 5: Trypan blue dye exclusion test in hepatocytes *(Source: https://www.google.com/)*.

Fluorescent Dye

A "fluorescent dye" emits light on excitation at a particular wavelength when it transitions from a higher electronic state to a lower electronic state within a short duration. This is a quick, simultaneous, twofold staining procedure utilizing fluorescein diacetate (FDA) and propidium iodide (PI), and is best considered as a beneficial, advantageous and dependable technique to choose reasonability and can be observed by flow cytometry or fluorescence microscopy. Certain fluorescent dyes are more dependable indicators of cell viability than the other conventional colored dyes. Viability assays utilizing PI with FDA are scored with the assumption that suitable live cells have strong esterase activity inside the cytoplasm and dead or dying cells demonstrate almost no esterase activity.

Lactate Dehydrogenase Leakage (LDH) Assay

LDH is a cytoplasmic protein that is available in all cells and is discharged into extracellular space when the plasma membrane is damaged.[61] The leakage of LDH into the cell culture medium can be recognized using tetrazolium. In the initial step, LDH produces reduced nicotinamide adenine dinucleotide (NADH) when it catalyzes the oxidation of lactate to pyruvate. In the second step, a tetrazolium salt is changed over to a chromogenic formazan product utilizing newly synthesized NADH in the presence of an electron acceptor.[62] The total formazan created can be colorimetrically evaluated by standard spectroscopy. However, there are some drawbacks; serum can contaminate the system with endogenous enzymes and mask the low levels of leached enzymes, and the presence of phenol red in the medium also masks the appearance of colored products of the enzyme reactions. Moreover, the MEM (minimum essential medium) lacks added pyruvate and therefore favors the lactate to pyruvate specific reaction.

LDH leakage has been compared to DNA leakage, as an endpoint in cytotoxicity testing, where it was found that the LDH leakage gave lower LC_{50} values (concentration causing 50% lethality) than DNA leakage.[63] This was suggested because to exhibit DNA leakage, the cells have to be more damaged compared with that for LDH to be lost. Thus, DNA leakage would not be a sensitive endpoint for cell damage/death.

Annexin V-FITC Assay

Annexin V is a 35-36 kDa, calcium-dependent, phospholipid-binding protein with a high affinity for phospholipid phosphatidylserine [PS]. In 1990, a protein vascular anticoagulant was found by Andree and his coworkers and was later renamed as Annexin V, which was limited to phospholipid bilayers in a calcium-dependent manner. In 1992, Fadok et al. established that macrophages sense PS that is exposed on the surface of the cells. A flow-cytometric test utilizes annexin V conjugated to FITC to gauge Annexin V binding to apoptotic cells.[64] Changes in the plasma membrane are one of the fundamental characteristics of the apoptotic cells. Phospholipids of the cell membrane are asymmetrically sandwiched between the inner and outer membrane phosphatidylcholine and sphingomyelin. While sphingomyelin is exposed to the external lipid bilayer, phosphatidylserine is positioned on the inner layer. During apoptosis, the symmetry is disturbed and the phosphatidylserine progresses toward appearing on the outer layer on the plasma membrane. Since the anticoagulant protein Annexin V binds with high affinity to phosphatidylserine, fluorochrome-conjugated Annexin V can be utilized to recognize apoptotic cells by flow cytometry. Figure 6 represents the schematics of Annexin V-FITC Assay.

FUNCTIONAL ASSAY

Tetrazolium Reduction Assays

A variety of tetrazolium compounds served as a milestone and offered a non-radioactive alternative to tritiated thymidine incorporation into DNA as a means of measuring cell proliferation. This group of compounds includes MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), and WST (water-soluble tetrazolium salts).[41,65-67]

The MTT tetrazolium compound was first homogenous assay appropriate for high throughput screening (HTS) as described by Mosmann in 1983. In the viable cells, reduction of yellow MTT to insoluble formazan crystals occurs that accumulates inside the cells and is stored close to the cell surface, as well as in the culture medium.[68] The formazan must be solubilized to read the absorbance that is associated with a viable cell number. The structure of MTT and the colored formazan is presented in Figure 7.

There are a few limitations of the MTT Assay:

- The absorption methodology in use is generally less sensitive than fluorescent and luminescent methods for detecting cell viability.
- Exposure of the MTT assay reagent to light and increased hydrogen ion concentration of the culture medium are also reported during the production of formazan and result in enhanced absorbance. Proper controls are required to investigate interference in assay chemistry.

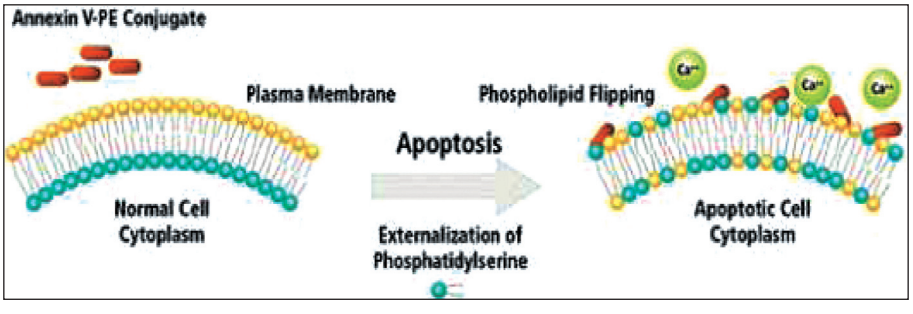

FIGURE 6: Annexin V - an overview *(Sources:http://www.bdbiosciences.com/br/instruments/accuri/articles/archive/2015_02/index.jsp).*

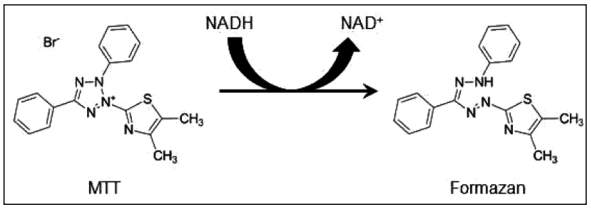

FIGURE 7: Structures of MTT and colored formazan product.

■ Riss and his colleagues (2013) reported MTT to be toxic for eukaryotic cells. Though extensively employed, it causes a certain degree of cytotoxicity during the treatment. Shortly after the addition of MTT, changes in the morphology of certain types of cells are observed. The changes in NIH 3T3 cell morphology after exposure to MTT are shown in Figure 8.Diverting the coenzyme NADH from the cellular functions toward the reduction of MTT into a colored formazan may potentially have an unfavorable outcome on cell health.

■ When MTT was developed, it was believed that mitochondrial succinate dehydrogenase is linearly related to the MTT reduction.[68] However, the latest research discredited the dogma that MTT is constantly diminished in the mitochondria.[69,70] Accordingly, studies also reflect that the cofactor NADH, which is needed for the MTT reduction is present not only in the mitochondria, but also in the cytoplasm, lysosomal membrane, and plasma membrane.

■ The original research of MTT assay involved the use of acidified isopropanol to solubilize the formazan crystals which benefits the conversion of phenol red to yellow with little interference in the absorbance. Various studies employed several solubilizing solutions like DMSO, dimethylformamide, SDS, a combination of detergents, and organic solvents.[71-73] Though detergent and solvent combinations stabilize, the absorbance value may lead to bubble formation and result in data variations.

ATP Bioluminescence Assay

The most reliable and widely used alternative to the MTT Assay is the ATP bioluminescence assay, which quantifies ATP levels and detects live, metabolically active dynamic cells. ATP has an adenine base, a ribose sugar, and three phosphate groups, which are bound by high energy bonds. These bonds discharge energy when cleaved and this energy is exploited to measure ATP bioluminescence.

This assay is based on an ATP luminescence response found in the firefly. In firefly, there are two compounds, Luciferin and Luciferase, which react in the presence of ATP to produce bioluminescence. The measure of bioluminescence is assessed by the Luminometer and is expressed in RLU (Relative Light Units). Since the RLU numbers correspond to the amount of ATP; this serves as an efficient marker of cell viability and metabolism. This process is illustrated in Figure 9. This assay is not suitable for spore detection because the level of ATP is low in

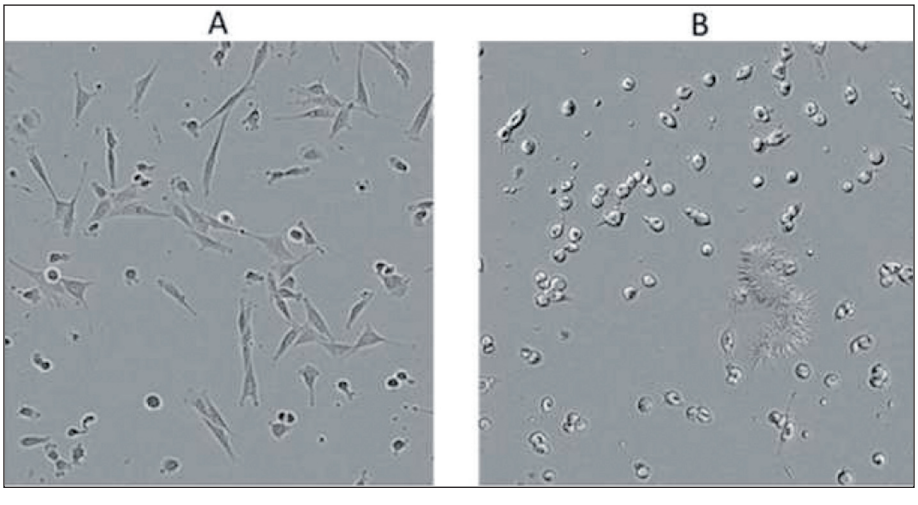

FIGURE 8: Changes in NIH 3T3 cell morphology after exposure to MTT *(Source: https://www.google.com/).*

$$\text{Luc} + \text{D-LH}_2 + \text{ATP} \xrightleftharpoons{Mg^{2+}} \text{Luc·LH}_2\text{-AMP} + PP_i \quad \textbf{(Eq. 1)}$$

$$\text{Luc·LH}_2\text{-AMP} + O_2 \rightarrow \text{Luc·Oxyluciferin}^* + \text{AMP} + CO_2 \quad \textbf{(Eq. 2)}$$

$$\text{Luc·Oxyluciferin}^* \rightarrow \text{Luc·Oxyluciferin} + \text{light} \quad \textbf{(Eq. 3)}$$

$$\text{Luc} + \text{L} + \text{ATP} \xrightarrow{Mg^{2+}} \text{Luc·L-AMP} + PP_i \quad \textbf{(Eq. 4)}$$

FIGURE 9: ATP-based bioluminescence found in firefly.

spores. Fujinami et al. (2004) demonstrated that a short incubation of the sample in the supplemented broth medium containing L-alanine increased RLU from spores and streamlined the ATP bioluminescent assay.[74]

Crystal Violet/Cell Adhesion Assay

Adherent cells detach from culture plates after their death. This attribute can be used for the indirect measurement of cell death and to assess contrasts in multiplication stimulation with death-inducing agents. One simple technique to distinguish adherence is the staining of the attached cells with crystal violet dye, hexamethyl pararosaniline chloride or pyocyanin, which bind to protein and DNA. The cells that experience death lose their adherence and are detached from live cells, decreasing the amount of crystal violet in a culture. The dye uptake by a viable cell is measured colorimetrically soon after acetic acid dye elution.

The Neutral Red Uptake Assay

The neutral red uptake test stands out among the most generally utilized cytotoxicity tests, developed at the Rockefeller University as a cell viability chemo-sensitivity assay. This assay depends on the aggregation of neutral red (NR) (3-amino-7-dimethyl-2-methylphenazine hydrochloride) in the lysosomes of viable cells. The NR dye does not accumulate in the lysosomes of dead or cells. Consequently, the dye is then removed from the viable cells utilizing an acidified ethanol solution and the absorbance of the solubilized dye is measured using a spectrophotometer. The uptake of neutral red relies upon the cell's ability to keep up pH gradients, through the generation of ATP. At physiological pH, the net charge of the dye is near zero, facilitating it to enter the membrane of the cell. Inside the lysosomes, there is a proton gradient to keep the pH lower than that of the cytoplasm. Subsequently, the dye gets charged and is held inside the lysosomes. At the point when the cell dies or the pH gradient is reduced, the dye cannot be held. Finally, the measure of retained dye is proportional to the number of viable cells. As indicated by the specificity of the lysosomal limit concerning the uptake of the dye, it is possible to recognize viable, damaged, or dead cells.

DNA LABELING ASSAY

Fluorescent Conjugates-TUNEL Assay

Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay is intended for distinguishing DNA fragmentation that results from apoptotic signaling cascades, by marking the 3' OH ends in the double-strand DNA breaks. Apoptosis is completed by cytosolic chemicals called caspases, which can degrade proteins to break down the cell, and activates another enzyme called caspase-activated DNase or CAD. This activated DNase cleaves the chromosomal DNA with nicked ends. The two fundamental TUNEL reagents, namely, terminal deoxynucleotidyltransferase or TdT and deoxyuridine triphosphate or dUTP, fluorescently label these nicked ends for recognition utilizing fluorescence microscopy, confocal microscopy or flow cytometry.[75,76]

Comet Assay

The comet Assay (single-cell gel electrophoresis) is a flexible and sensitive strategy for estimating single and double-strand breaks in DNA at the level of individual cells. This assay gets its name from a characteristic comet-like appearance: a splendid bright head with intact DNA and a tail with damaged and fragmented DNA.[77,78] This technique was originally developed by Ostling and Johansson in 1984 and later modified by Singh et al. in 1988 as the "Alkaline Comet Assay".[79,80] After exposure to the test reagents, the cells are embedded in thin agarose gels on microscopic slides. Proteins are washed away by cell-lysis. A typical hydrogen ion concentration maintained during DNA unwinding

and electrophoresis plays a crucial role in determining the type of damage. A neutral pH condition predominantly facilitates the detection of double-strand DNA breaks, while basic pH conditions permit the identification of single-strand breaks and express alkali-labile sites and other repair sites. When an electric field is applied, the DNA, owing to its negative charge, is drawn toward the positively charged anode. The total amount of DNA that leaves the cavity gives an estimate of the damaged DNA.

ASSAYS BASED ON MORPHOLOGIES

Microscopic Observation

Light microscopy

In the early stages of apoptosis, a range of morphological changes like cell shrinkage and pyknosis or nuclear condensation takes place. A proper staining method is a prerequisite to assess apoptotic changes. Hematoxylin is a positively charged dark blue stain, binds to basophilic substances such as DNA/RNA, and stains them violet. Eosin is a negatively charged red/pink stain, which binds to acidophilic substances, for example, amino acids and proteins and stains them pink. Hence, the following structures can be observed in optical microscopy: nuclei in blue/purple, cytoplasm in red, muscles in dark red, collagen and mitochondria in pale pink, erythrocytes in cherry red and basophils in purple-red. An experienced technician is needed to properly differentiate these because the mitotic cells at the early stage are confused with apoptotic cells.

Electron microscopy

Electron Microscopy (EM) is viewed as the “gold standard” for the detection of apoptotic changes in cells. However, the procedure is time-consuming (transmission electron microscopy, TEM, analysis consumes 5-6 days; scanning electron microscopy, SEM, requires 24 h) and expensive. Nevertheless, the plethora of information provided is wide, while TEM investigation is essentially qualitative, whereas SEM studies can give information on the cell surface, cell-cell and cell-substrates interactions, but it is difficult to evaluate the apoptotic features through SEM.

Certain disadvantages in EM observation limits its usage,

1. Many samples cannot be analyzed at one time.

2. The sample preparation process is labor-intensive.

3. Only a small area can be visualized at a time; therefore, the extent of apoptosis is difficult to estimate.

Video optical microscopy

The cells which undergo apoptosis are optionally observed in phase contrast mode and recording is made through a camera which then connected to a video recorder.[81,82] This type of assessment is better known for the documentation of apoptosis-associated cellular changes in the time course of the event. The differences in the changes seen in apoptosis and necrosis are well documented.[82] This type of analysis is highly useful in recording the sequence of morphological changes observed after introducing apoptosis-inducing agents to cell culture media.

Atomic Force Microscopy (AFM)

The principal task of the AFM is fundamentally the same as that of a stylus profilometer, a sharp cantilever tip interfaces with the sample surface identifying the local forces between the atoms of the tip and the test surface. The trademark property of the AFM is that the pictures are obtained by 'feeling' the sample surface without using light. In this way, not just the sample topography can be recorded with great resolution, the material attributes and the strength of communication between the sample surface and the cantilever tip can also be estimated. AFM offers much detail as possibly offered by optical microscopy. It is very peculiar in imaging cellular surface; therefore, this technique is frequently employed in studying cells undergo apoptosis.[83]

Hessler presented a theory in which human epidermoid carcinoma cell line KB, when treated with staurosporine, showed that the cell height was diminished by 32%, the cell volume was decreased by 50%, and cellular diameter was changed by nearly 15%. Thus, AFM is deemed to be an ideal technique when coupled with other imaging techniques to investigate the apoptotic pathways.

REPRODUCTIVE ASSAY

Colony Forming Efficiency

A clonogenic assay or a colony-forming efficiency assay is the most reliable cell biological technique to detect colony-forming ability and is employed both in research as well as for determining cytotoxicity of a chemical compound. This basic tool was described in 1955 for studying the effect of radiation of cells. This method measures the number of colonies derived from single cells. Normally, a few hundred cells are inoculated. After an appropriate incubation time of 5 to 8 days, each viable cell forms colonies, which are stained and checked physically using a colony counter.

A cluster of blue-stained cells, which comprise at least 25-50 cells are considered as a colony. An average of three colony counts and the mean divided by the number of cells plated gives the plating efficiency (PE).

$$PE = \frac{\text{Number of colonies counted}}{\text{Number of cells plated}} \times 100$$

Following the determination of PE, surviving fraction (SF) of cells is also estimated as:

$$SF = \frac{\text{PE of treated sample}}{\text{PE of control}} \times 100$$

CONCLUSION

The cells of a multicellular organism are members of a highly organized community. The quantity of cells in this community is firmly regulated - not just by controlling the rate of cell division but also by controlling the rate of cell death. This procedure is called apoptosis (programmed cell death). Apoptosis is a type of cell death managed through a precise pathway by a progression of signal cascades under specific circumstances. It assumes a basic role in directing development, advancement and immune response, and clearing redundant or abnormal cells in organisms. It is also an important means by which organisms can maintain a constant number of cells to live successfully. If the cells are not required again, they commit suicide by initiating an intracellular death program. Apoptosis plays an important role in sculpting the shape and organization of organs during development by eliminating specific populations of cells at different stages of embryogenesis. Apoptosis is a well-regulated process in which energy is required; it is characterized by morphological and biochemical changes, where caspase activation plays a significant role. Although a significant number of the key apoptotic proteins are activated or inactivated in the apoptotic pathways, the exact mechanisms of action of these proteins have not yet been fully understood. Understanding the machinery of apoptosis is essential because programmed cell death is initiated by physiological and pathological factors, and would concurrently provide deeper insight into various disease processes and may influence therapeutic strategy.

The measure of apoptosis that happens in tissues of growing and adult organisms can be shocking. In a healthy grown-up human, billions of cells die in the bone marrow and digestive tract consistently. It appears to be strikingly inefficient for such huge numbers of cells to die, particularly as by far most are healthy at the time they commit suicide. In multicellular life forms, cells that are never required again or the ones which may be a threat to the organism are destroyed by a firmly managed cell suicide process as apoptosis. Apoptosis is carried out by proteolytic enzymes called caspases, which trigger cell death by cleaving specific proteins in the cytoplasm and nucleus. Caspases exist in all cells as dormant precursors or procaspases, which are generally activated through cleavage by other caspases, creating a cascade of proteolytic caspases. This activation procedure is initiated by either extracellular or intracellular death signals, which prompt intracellular adaptor molecules to accumulate and initiate procaspases. The induction and execution of apoptosis require the cooperation of a progression of molecules including signal particles, receptors, chemicals, and gene directing proteins. The underlying apoptotic pathways and how they are dysregulated in cancer, in addition to our ability to design effective therapies targeting such mechanisms, should be deeply investigated. De-

ficient apoptosis is related to tumors, issues of the immune system like autoimmune disorders and viral contaminations, while excessive apoptosis is related to ischemic damage, for example, myocardial infarction, stroke, AIDS, neurodegenerative diseases, sepsis, and multiple organ dysfunction syndromes. Apoptosis is responsible for cell death, tissue homeostasis and atrophy initiated by endocrine and other stimuli, negatively affects the immune system framework and the generous extent of T-cell killing. Although dysregulated apoptosis in aggressive malignant cells remains an attractive focus in tumor treatment, much work is required to understand the maximum capacity of such methodologies.

Despite many methodologies of apoptotic detection, such as membrane integrity assay, functional and reproductive assay, probe binding techniques and advance microscopy, several challenges still remain. Optimization, better resolution, and sensitivity remain an important issue to focus on. Novel analytical techniques involving gene editing (CRISPR-Cas9) must be continually introduced so that the apoptotic process can be better understood. Moreover, accurate detection of apoptosis enables the calculation of apoptotic index which could thereby predict the outcome of the treatment.

Acknowledgement

One of the Authors, Suganya, gratefully acknowledges the University Grants Commission, Government of India, New Delhi for providing fellowship through UGC-BSR Scheme and Madurai Kamaraj University, Madurai for providing UGC-UPE Non-NET fellowship. Prof. Dr. K. Muthuchelian would like to gratefully acknowledge UGC-UPE, UGC-BSR One Time Grant and the support of Dayananda Sagar University during the execution of the manuscript.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Krishnasamy Muthuchelian;* ***Design:*** *Krishnasamy Muthuchelian, Suganya Chinnasamy;* ***Control/Supervision:*** *Krishnasamy Muthuchelian;* ***Data Collection and/or Processing:*** *Suganya Chinnasamy, Farhan Zameer;* ***Analysis and/or Interpretation:*** *Suganya Chinnasamy, Krishnasamy Muthuchelian;* ***Literature Review:*** *Suganya Chinnasamy;* ***Writing the Article:*** *Krishnasamy Muthuchelian, Suganya Chinnasamy, Farhan Zameer;* ***Critical Review:*** *Krishnasamy Muthuchelian;* ***References and Fundings:*** *Krishnasamy Muthuchelian;* ***Materials:*** *Suganya Chinnasamy.*

REFERENCES

1. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. Br J Cancer. 1972;26(4):239-257. [Crossref] [PubMed] [PMC]
2. Armanios M, Blackburn EH. The telomere syndromes. Nat Rev Genet. 2012;13(10):693-704. [Crossref] [PubMed] [PMC]
3. Eisenberg DT. An evolutionary review of human telomere biology: the thrifty telomere hypothesis and notes on potential adaptive paternal effects. Am J Hum Biol. 2011;23(2):149-167. [Crossref] [PubMed]
4. Aubert G, Lansdorp PM. Telomeres and aging. Physiol Rev. 2008;88(2):557-579. [Crossref] [PubMed] [PMC]
5. Ornish D, Lin J, Chan JM, et al. Effect of comprehensive lifestyle changes on telomerase activity and telomere length in men with biopsy-proven low-risk prostate cancer: 5-year follow-up of a descriptive pilot study. Lancet Oncol. 2013;14(11):1112-1120. [Crossref] [PubMed]
6. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer. 2002;2(4):277-288. [Crossref] [PubMed]
7. Martinvalet D, Zhu P, Lieberman J. Granzyme A induces caspase- independent mitochondrial damage, a required first step for apoptosis. Immunity. 2005;22(3):355-370. [Crossref] [PubMed]
8. Hengartner MO. The biochemistry of apoptosis. Nature. 2000;407(6805):770-776. [Crossref] [PubMed]
9. Cohen GM. Caspases: the executioners of apoptosis. Biochem J. 1997;326(pt 1):1-16. [Crossref] [PubMed] [PMC]
10. Rai NK, Tripathi K, Sharma D, Shukla VK. Apoptosis: a basic physiologic process in wound healing. Int J Low Extrem Wounds. 2005;4(3):138-144. [Crossref] [PubMed]
11. Hu S, Snipas SJ, Vincenz C, Salvesen G, Dixit VM. Caspase-14 is a novel developmental regulated protease. J Biol Chem. 1998;273 (45):29648-29653. [Crossref] [PubMed]
12. Nakagawa T, Zhu H, Morishima N, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature. 2000;403(6765):98-103. [Crossref] [PubMed]

13. Koenig U, Eckhart L, Tschachler E. Evidence that caspase-13 is not a human but a bovine gene. Biochem Biophys Res Commun. 2001;285(5):1150-1154. [Crossref] [PubMed]
14. Kang SJ, Wang S, Kuida K, Yuan J. Distinct downstream pathways of caspase-11 in regulating apoptosis and cytokine maturation during septic shock response. Cell Death Differ. 2002;9(10):1115-1125. [Crossref] [PubMed]
15. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. 2001;104(4):487-501. [Crossref] [PubMed]
16. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science. 1998;281 (5381):1305-1308. [Crossref] [PubMed]
17. Chicheportiche Y, Bourdon PR, Xu H, et al. TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. J Biol Chem. 1997;272(51):32401-32410. [Crossref] [PubMed]
18. Peter ME, Krammer PH. Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. Curr Opin Immunol. 1998;10(5):545-551. [Crossref] [PubMed]
19. Suliman A, Lam A, Datta R, Srivastava RK. Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and -independent pathways. Oncogene. 2001;20(17): 2122-2133. [Crossref] [PubMed]
20. Rubio-Moscardo F, Blesa D, Mestre C, et al. Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. Blood. 2005;106(9): 3214-3222. [Crossref] [PubMed]
21. Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell. 1995;81(4):495-504. [Crossref] [PubMed]
22. Grimm LM, Alfred LG, Guy G, et al. Proteasomes play an essential role in thymocyte apoptosis. The EMBO Journal. 1996;15(15): 3835-3844. [Crossref] [PubMed] [PMC]
23. Wajant H. The Fas signaling pathway: more than a paradigm. Science. 2002;296(5573): 1635-1636. [Crossref] [PubMed]
24. Kischkel FC, Hellbardt S, Behrmann I, et al. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. 1995;14(22):5579-5588. [Crossref] [PubMed] [PMC]
25. Kataoka T, Schröter M, Hahne M, et al. FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation. J Immunol. 1998;161(8):3936-3942. [PubMed]
26. Scaffidi C, Schmitz I, Krammer PH, Peter ME. The role of c-FLIP in modulation of CD95- induced apoptosis. J Biol Chem. 1999;274(3): 1541-1548. [Crossref] [PubMed]
27. Hitoshi Y, Lorens J, Kitada SI, et al. Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells. Immunity. 1998;8(4):461-471. [Crossref] [PubMed]
28. Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P. Toxic proteins released from mitochondria in cell death. Oncogene. 2004;23(16):2861-2874. [Crossref] [PubMed] [PMC]
29. Cai J, Yang J, Jones DP. Mitochondrial control of apoptosis: the role of cytochrome c. Biochim Biophys Acta. 1998;1366(1-2):139-149. [Crossref] [PubMed]
30. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. 2000;102(1): 33-42. [Crossref] [PubMed] [PMC]
31. Loo D. TUNEL assay. An overview of techniques. Methods Mol Biol. 2002;(203):21-30. [Crossref] [PubMed]
32. Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G. Mechanisms of cytochrome c release from mitochondria. Cell Death Differ. 2006;13(9):1423-1433. [Crossref] [PubMed]
33. Chinnaiyan AM. The apoptosome: heart and soul of the cell death machine. Neoplasia. 1999;1(1):5-15. [Crossref] [PubMed] [PMC]
34. Hill MM, Adrain C, Duriez PJ, Creagh EM, Martin SJ. Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. EMBO J. 2004;23(10):2134-2145. [Crossref] [PubMed] [PMC]
35. van Loo G, van Gurp M, Depuydt B, et al. The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. Cell Death Differ. 2002;9(1):20-26. [Crossref] [PubMed]
36. Schimmer AD. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res. 2004;64(20):7183-7190. [Crossref] [PubMed]
37. Ekert PG, Vaux DL. The mitochondrial death squad: hardened killers or innocent bystanders? Curr Opin Cell Biol. 2005;17(6):626-630. [Crossref] [PubMed]
38. Joza N, Susin SA, Daugas E, et al. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature. 2001;410(6828):549-554. [Crossref] [PubMed]
39. Susin SA, Daugas E, Ravagnan L, et al. Two distinct pathways leading to nuclear apoptosis. J Exp Med. 2000;192(4):571-580. [Crossref] [PubMed] [PMC]
40. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature. 2001;412(6842):95-99. [Crossref] [PubMed]
41. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer. 2002;2(9):647-656. [Crossref] [PubMed]
42. Schuler M, Green DR. Mechanisms of p53-dependent apoptosis. Biochem Soc Trans. 2001;29(pt 6):684-688. [Crossref] [PubMed]
43. Liu FT, Newland AC, Jia L. Bax conformational change is a crucial step for PUMA-mediated apoptosis in human leukemia. Biochem Biophys Res Commun. 2003;310(3):956-962. [Crossref] [PubMed]
44. Oda E, Ohki R, Murasawa H, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53- induced apoptosis. Science. 2000;288(5488):1053-1058. [Crossref] [PubMed]
45. Shibue T, Suzuki S, Okamoto H, et al. Differential contribution of Puma and Noxa in dual regulation of p53-mediated apoptotic pathways. EMBO J. 2006;25(20):4952-4962. [Crossref] [PubMed] [PMC]
46. Chen L, Willis SN, Wei A, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell. 2005;17(3):393-403. [Crossref] [PubMed]
47. Meyer N, Kim SS, Penn LZ. The Oscar-worthy role of Myc in apoptosis. Semin Cancer Biol. 2006;16(4):275-287. [Crossref] [PubMed]
48. Slee EA, Adrain C, Martin SJ. Executioner caspase-3, -6, and -7 perform distinct, nonredundant roles during the demolition phase of apoptosis. J Biol Chem. 2001;276(10):7320-7326. [Crossref] [PubMed]
49. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol. 1992;148(7):2207-2216. [PubMed]
50. Martin SJ, Green DR. Protease activation during apoptosis: death by a thousand cuts? Cell. 1995;82(3):349-352. [Crossref] [PubMed]
51. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest. 1998;101(4):890-898. [Crossref] [PubMed] [PMC]
52. Cadena-Herrera D, Esparza-De Lara JE, Ramírez-Ibañez ND, et al. Validation of three viable-cell counting methods: manual, semi-automated, and automated. Biotechnol Rep (Amst). 2015;(7):9-16. [Crossref] [PubMed] [PMC]

53. Philpott NJ, Turner AJ, Scopes J, et al. The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. Blood. 1996;87(6):2244-2251. [Crossref] [PubMed]
54. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 1997;A3.B.1-A3.B.2.
55. Lecoeur H. Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases. Exp Cell Res. 2002;277(1):1-14. [Crossref] [PubMed]
56. Wilson JG, Beaudoin HJ. Free HJ. Studies on the mechanism of teratogenic action of trypan blue. Anat Rec. 1959;133(2):115-128. [Crossref] [PubMed]
57. Barber AN, Geer JC. Studies on the teratogenic properties of trypan blue and its components in mice. J Embryol Exp Morphol. 1964;(12):1-14. [PubMed]
58. Beck F, Lloyd JB. Dose-response curves f or the teratogenic activity of trypan blue. Nature. 1964;(201):1136-1137. [Crossref] [PubMed]
59. Turbow MM. Teratogenic effect of trypan blue on rat embryos cultivated in vitro. Nature. 1965;206(984):637. [Crossref] [PubMed]
60. Field FE, Roberts G, Hallowes RC, Palmer AK, Williams KE, Lloyd JB. Trypan blue: identification and teratogenic and oncogenic activities of its coloured constituents. Chem. Biol Interact. 1977;16(1):69-88. [Crossref] [PubMed]
61. Burd JF, Usategui-Gomez M. A colorimetric assay for serum lactate dehydrogenase. Clin Chim Acta. 1973;46(3):223-227. [Crossref] [PubMed]
62. Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods. 1983;64(3):313-320. [Crossref] [PubMed]
63. Skaanild MT, Clausen J. Estimation of LC5q values by assay of lactate dehydrogenase and DNA redistribution in human lymphocyte cultures. ATLA. 1989;16:293-296.
64. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood. 1994;84(5): 1415-1420. [Crossref] [PubMed]
65. Tominaga H, Ishiyama M, Ohseto F, et al. A watersoluble tetrazolium salt useful for colorimetric cell viability assay. Anal Commun. 1999;36(2):47-50. [Crossref]
66. Ishiyama M, Shiga M, Sakamoto K, Mizoguchi M, He PG. A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. Chem Pharm Bull. 1993;41(6):1118-1122. [Crossref]
67. Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun. 1991;3(7):207-212. [Crossref] [PubMed]
68. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55-63. [Crossref] [PubMed]
69. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Annu Rev. 2005;(11):127-152. [Crossref] [PubMed]
70. Bernas T, Dobrucki J. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry. 2002;47(4):236-242. [Crossref] [PubMed]
71. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods. 1989;119(2):203- 210. [Crossref] [PubMed]
72. Tada H, Shiho O, Kuroshima K, Koyama M, Tsukamoto K. An improved colorimetric assay for interleukin 2. J Immunol Methods. 1986;93(2):157-165. [Crossref] [PubMed]
73. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 1986;89(2):271-277. [Crossref] [PubMed]
74. Fujinami Y, Kataoka M, Matsushita K, et al. Sensitive detection of bacteria and spores using a portable bioluminescence ATp measurement. Assay system distinguishing from white powder materials. Journal of Health Science. 2004;50(2):126-132. [Crossref]
75. Lozano GM, Bejarano I, Espino J, et al. Density gradient capacitation is the most suitable method to improve fertilization and to reduce DNA fragmentation positive spermatozoa of infertile men. Anatolian Journal of Obstetrics & Gynecology. 2009;3(1):1-7.
76. Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z. Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sper cells. Analogy to apoptosis of somatic cells. Exp Cell Res. 1993;207(1):202-205. [Crossref] [PubMed]
77. Nandhakumar S, Parasuraman S, Shanmugam MM, Rao KR, Chand P, Bhat BV. Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay). J Pharmacol Pharmacother. 2011;2(2):107-111. [Crossref] [PubMed] [PMC]
78. Tice RR, Agurell E, Anderson D, et al. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 2000;35(3):206-221. [Crossref] [PubMed]
79. Ostling O, Johanson KJ. Single-cell gel electrophoresis assay. Biochem Biophys Res Commun. 1984;(123):291-298. [Crossref]
80. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175(1):184-191. [Crossref] [PubMed]
81. Willingham MC. Cytochemical methods for the detection of apoptosis. J Histochem Cytochem. 1999;47(9):1101-1109. [Crossref] [PubMed]
82. Collins JA, Schandl CA, Young KK, Vesely J, Willingham MC. Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem. 1997;45(7):923-934. [Crossref] [PubMed]
83. Hessler JA, Budor A, Putchakayala K, et al. Atomic force microscopy study of early morphological changes during apoptosis. Langmuir. 2005;21(20):9280-9286. [Crossref] [PubMed]