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Review Article

Review on mechanisms of regulating apoptosis in animal cells

Yobsan Tamiru[1]*, Nigus Abebe[2] and Abriham Kebede[1]

[1]*School of Veterinary Medicine, College Of Medical And Health Science, Wollega University, P.O. Box, 395, Nekemte, Ethiopia*
[2]*College of Veterinary Medicine, Mekelle University, Ethiopia*

ABSTRACT

In normal tissues, cell proliferation is generally restricted to cells that replenish the tissue. Cell number is dependent not only on cell proliferation, but also on cell death. Apoptosis is the process by which excess or damaged cells in the body are removed. This is an energy dependent a synchronic process that comprises the loss of cell-to-cell contacts, cytoplasmic shrinkage, membrane blebbing, DNA fragmentation, disassembly of the nuclei and formation of apoptotic bodies. The mechanism of apoptosis is highly complex and involves energy dependent cascade of molecular events. It is mediated mainly through three pathways: extrinsic, intrinsic and perforin pathway. The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. Proper function of the apoptotic machinery is of fundamental importance during the growth and development of the organism, because apoptosis in accord with cell division ensures the proper shaping and the structural and functional integrity of the various tissues and organs. Morphological features of apoptosis can be detected by various cytochemical and microscopic methods.

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*** E-mail:** abrahamkebede2016@gmail.com, yobsanraji@gmail.com

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1. INTRODUCTION

In normal tissues, cell proliferation is generally restricted to cells that replenish the tissue. Most tissues are thought to contain stem cells that have this replenishment function. Stem cells are self-renewing cells that can divide asymmetrically to yield a new stem cell and a progenitor cell. Progenitor cells may or may not undergo further divisions, ultimately leading to terminal differentiation. Once cells have terminally differentiated, they have a specialized function and are no longer dividing. Most tissues are made up of such non dividing cells. Thus proliferation is normally tightly controlled so that only particular cells in the body are dividing [1].

Cell number is dependent not only on cell proliferation, but also on cell death. Plants, animals and several unicellular eukaryotes use programmed cell death (PCD) for defense and developmental mechanisms. There are likely to be inherent differences in the operational mechanism of PCD between plants and animals. Although, very few regulatory proteins or protein domains have been identified as conserved across all eukaryotic PCD forms, still plants and animals share many hallmarks of PCD, both at cellular and molecular levels. Morphological and biochemical features like chromatin condensation, nuclear DNA fragmentation and participation of caspase like proteases in plant PCD appear to be similar across the eukaryotic kingdom and in conformity with the process in metazoans as well [2].

PCD or apoptosis is the process by which excess or damaged cells in the body are removed. Moreover, it is the balance between the production of new cells and cell death that maintains the appropriate number of cells in a tissue [3].
Apoptosis is an essential life process for all cellular organisms. In all animals, regulated cell death plays key roles in a variety of biological processes ranging from embryogenesis to

immunity. It is also an active process of cellular self-destruction that plays an important role in a large number of disorders, including organ rejection after transplantation [4], myocardial ischemia or infarct and apoptotic cells have also been observed during tumor growth and regression. Thus, for example, apoptotic bodies of epithelial cell origin were described during development of squamous cell carcinoma [5].

As indicated in Figure 1, apoptosis is in contrast to the necrotic mode of cell-death in which case the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disrupture of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue [6].

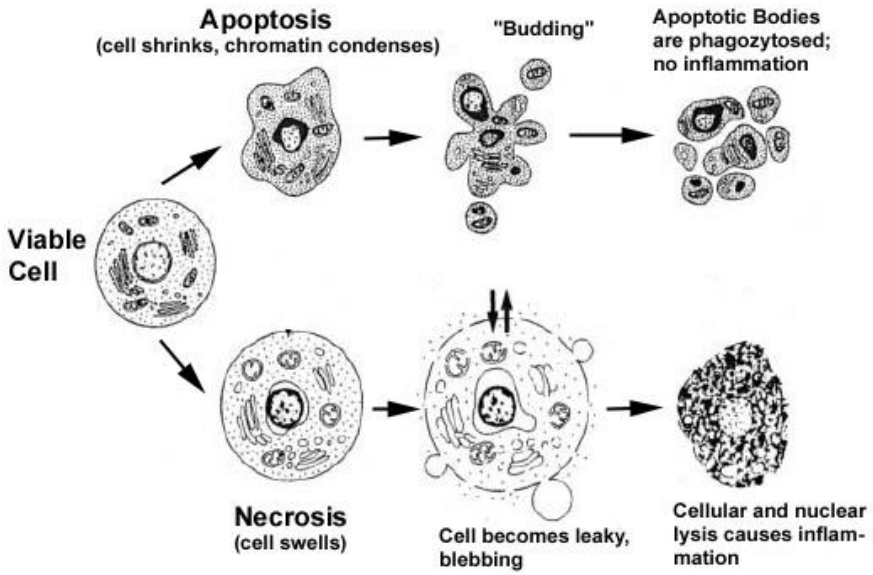

Figure1. Comparative morphology of apoptotic and necrotic cells [45].

Apoptotic cells can be recognized by stereotypical morphological changes such as the cell shrinks, shows deformation and looses contact to its neighboring cells. Its chromatin condenses and marginates at the nuclear membrane and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies'. These apoptotic bodies are recognized, engulfed, and degraded by professional phagocytes and innate immune cells and thus are removed from the tissue without causing an inflammatory response [7].

Alterations in regulation of the death process could result in retardation or absence of death, leaving supernumerary cells open to the possibility of forming new cellular interactions, acquiring new functions, and facing new demands [8].

Therefore the objective of this paper is to highlight the mechanism and role of apoptosis in animal cells.

2. MORPHOLOGICAL ALTERATIONS IN APOPTOSIS

2.1. Nuclear alterations

Morphological hallmark of apoptosis in nucleus are chromatin condensation and nuclear fragmentation which are mainly because of different enzymes and solutes released [9].

2.2. Alteration in cell membrane and cytosol

Initially during apoptosis, cell detaches from its substratum and adjacent cells. Membrane and organelles are well preserved. Subsequently cells start to show extensions or protuberances of plasma membrane commonly referred to as blebs. Following cell shrinkage, these blebs separate forming apoptotic bodies, which are round, smooth membrane bound remnants densely packed with organelles and nuclear fragments [10-11].

2.3. Mitochondrial Alterations

Mitochondrial membrane permeabilisation has a central role during apoptosis degradation cascade. Proapoptotic members of Bcl-2 family are involved in initiation of mitochondrial membrane Permeabilisation whereas anti apoptotic factors of Bcl-2 family inhibits this process. Moreover there is loss of transmembrane potential [9].

3. REGULATION MECHANISM OF APOPTOSIS

The mechanism of apoptosis is highly complex and involves energy - dependent cascade of

molecular events. It is mediated mainly through three pathways; namely extrinsic or death receptor pathway, intrinsic or mitochondrial pathway and perforin or granzyme pathway [12].

3.1. Extrinsic pathway

Extrinsic path way involves the initiation of apoptosis through ligation of plasma membrane death receptors and resulting in the recruitment of adaptor proteins which cause activation of several enzymes leading to apoptosis [13].

3.1.1. Caspases

Caspases are a class of cysteine-aspartyl proteases that are synthesized as inactive precursor enzymes or proenzymes. These proteases typically lie dormant in the healthy cell and in response to cell-death stimuli, are converted, either by proteolytic cleavage or by recruitment into large complexes, into active enzymes. Once activated, caspases cleave the substrates after conserved aspartate residues effects of caspases on cellular substrates bring about the morphological features of apoptosis [14].

3.1.2. Growth factor deprivation

Deprivation of growth factors leads to the induction of apoptosis, i.e. condensation of chromatin and degradation in oligonucleosomesized fragments, formation of plasma and nuclear membrane blebs and cell fragmentation into apoptotic bodies which can be taken up by neighboring cells [15]. A change in the pattern of gene expression in the cell will also occur. Recent experiments have demonstrated that an 'imbalance' in expression of genes involved in the stimulation of proliferation by growth factors may be responsible for entry into apoptosis upon factor removal [16].

3.1.3. Tumor necrosis factor (TNF) activation

The main function of the death domain is to transmit the external death signal from the cell's surface to the intracellular signaling pathways [17]. Following binding of the ligand to the corresponding receptor, cytoplasmic adapter proteins can exhibit the corresponding death domains. Ligation of the TNF ligand to the corresponding TNF receptor mediates its binding to TNF-receptor associated death domain, which in turn leads to recruitment of Fragment Associated Death Domain and receptor-interacting protein [18].

3.2. Intrinsic pathway

The intrinsic pathway also known as mitochondrial cell death pathway since apoptosis occurs secondary to imbalance in intracellular homeostasis. Following an apoptotic trigger, several apoptogenic proteins are released from mitochondria [19].

3.2.1. Mitochondrial regulations

Besides amplifying and mediating extrinsic apoptotic pathways, mitochondria also play a central role in the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation as well as those induced by chemotherapeutic drugs [20-21]. Most apoptosis-inducing conditions involve the disruption of the mitochondrial inner trans-membrane potential as well as the so called permeability transition, a sudden increase of the inner mitochondrial membrane permeability to solutes with a molecular mass below approximately 1.5KDa. Concomitantly, osmotic mitochondrial swelling has been observed by influx of water into the matrix with eventual rupture of the outer mitochondrial membrane, resulting in the release of proapoptotic proteins from the mitochondrial intermembrane space in to the cytoplasm [22]. Released proteins include cytochrome C, which activates the apoptosome and therefore the caspase cascade, but also other factors such as the apoptosis-inducing factor [23].

3.3. Perforin/Granzyme pathway

Cytotoxic cells induce in target cells morphological changes that trigger apoptosis. Apoptotic cells are in higher numbers in tumors that are strongly infiltrated with cytotoxic T lymphocytes or natural killer (NK) cells.T-cell mediated cytotoxicity is a variant of type IV hypersensitivity where sensitized CD8+ cells kill antigen-bearing cells. These cytotoxic T lymphocytes are able to kill target cells via the extrinsic pathway [24]. However, they are also able to exert their cytotoxic effects on tumour cells and virus-infected cells via a novel pathway that involves secretion of the transmembrane pore-forming molecule perforin with a subsequent exophytic release of cytoplasmic granules through the pore and into the target cell [25].

The serine proteases granzyme A and granzyme B are the most important component within the granules. The granzyme B can utilize the mitochondrial pathway for amplification of the death signal by specific cleavage and induction of cytochrome *c* release [26]. Granzyme A is also important in cytotoxic T cell induced apoptosis and activates caspase independent pathways. Once in the cell, granzyme A activates DNA nicking via DNAse specifically a tumour suppressor gene product. This DNAse has an important role in immune surveillance to prevent cancer through the induction of tumor cell apoptosis [27].

4. CLINICAL RELEVANCE OF APOPTOSIS

4.1. Viral clearance

Cells infected with a virus can undergo apoptosis as a defence mechanism to prevent

viral infection [28]. During acute viral infections, the number of virus-specific T cells, including CTLs increases resulting in apoptosis of virus-infected cells and viral clearance. For example, the appearance of $CD3^+$ CTLs coincides with a drop in Theiler's murine encephalomyelitis virus (TMev) titre during acute disease, which suggests that these cells are involved in the apoptotic death of TMeV-infected cells and the partial clearance of TMeV from the CNS [29]. CTLs secrete cytotoxic cytokines, including TNF and express high levels of surface Fas ligand (FasL) and tumour necrosis factor related inducing ligand, which can induce apoptosis by interacting with death receptors on target cells and activating the extrinsic apoptotic pathway [30]. In animal models of virus-induced disease, virus-infected cells thus appear to die by apoptosis in which this process can facilitate viral clearance. The central role of apoptosis in virus-induced tissue injury has prompted studies designed to test the effect of inhibition of apoptosis on both viral growth and the severity of virus-induced disease [31].

4.2. Cancer

Apoptosis can represent a defense mechanism at cellular level against cancer by the participation of protooncogenes and tumor suppressor genes in the regulation of apoptosis. Both normal apoptotic processes and normal cell mechanisms that control proliferation usually need to be altered to produce enough abnormal cell proliferation to cause cancer [32].

Cancer is usually envisaged as a disease of excessive cellular proliferation. Recently, genetic alterations that dysregulate the physiological cell death process appeared to contribute to the clonal expansion of malignant cells. Accordingly, a number of oncogenes and anti-oncogenes have been found to regulate apoptotic cell death. Oncogenes that promote cell proliferation and those that inhibit cell death could co-operate to induce a neoplastic lesion [33]. In fact, suppression of apoptosis during carcinogenesis is thought to play a central role in the development and progression of some cancers [34]. There are a variety of molecular mechanisms that tumour cells use to suppress apoptosis. Tumour cells can acquire resistance to apoptosis by the expression of anti-apoptotic proteins or by the down-regulation or mutation of pro-apoptotic proteins. The expression of these proteins is regulated by the *p53* tumor suppressor gene [35].

Another method of apoptosis suppression in cancer involves evasion of immune surveillance [36]. Certain immune cells (T cells and NK cells) normally destroy tumor cells via the perforin/granzyme B pathway or the death-receptor pathway. In order to evade immune destruction, some tumor cells will diminish the response of the death receptor pathway to FasL produced by T cells [37].

5. DETECTION OF APOPTOSIS

Morphological features of apoptosis can be detected by various cytochemical and microscopic methods. The use of inter calatory agents such as propidium iodide(PI) and Hoechst 3325 enables the detection of the condensation and marginalization of chromatin in the nucleus by measuring the level of fluorescence, which is reduced during apoptosis [38]. The following are among the techniques used for the detection of apoptosis in animal cells.

5.1. TUNEL Technique

Terminal Uridine Nucleotide End Labelling (TUNEL) staining was initially described as a method for staining cells that have undergone programmed cell death, or apoptosis, and exhibit the biochemical hallmark of apoptosis — internucleosomal DNA fragmentation. TUNEL staining relies on the ability of the enzyme terminal Deoxy nucleotidyl transferase to incorporate labeled dUTP into free 3'-hydroxyl termini generated by the fragmentation of genomic DNA into low molecular weight double-stranded DNA and high molecular weight single stranded DNA [39].
The principle of TUNEL assay relies on terminal deoxynucleotidyl transferase (TdT)-mediated addition of a modified dUTP (X-dUTP) to 3'-OH ends of DNA fragments that are generated as a result of apoptosis induction. TUNEL assays identify apoptotic cells by the terminal deoxynucleotidyl transferase (TdT)-mediated addition of labeled (X) deoxyuridine triphosphate nucleotides (X-dUTPs) to the 3'-OH end of DNA strand breaks that are subsequently visualized depending on the introduced label thus serving as parameter for the percentage of apoptotic cells within the analysed cell population. The assay sensitivity strongly depends on the incorporation efficiency of the modified dUTP that is influenced by size/bulkiness of the attached label .To avoid the loss of fragmented DNA and to allow enzyme and nucleotide entrance, cells need to be fixed and subsequently permeabilized prior to the labelling reaction. The TUNEL technique reaction was demonstrated by the presence of a golden brown coloration which showed the specific morphological characteristic of apoptotic cell [40].

5.2. Flow cytometry

Flow cytometry is a technique of quantitative single cell analysis. **I**t is a powerful tool for interrogating the characteristics of cells. It is based upon the light-scattering properties of the cells being analyzed and these include fluorescence emissions. This fluorescence may be associated with dyes specific for molecules either on the surface or in the intracellular components of the cell. Flow cytometry facilitates the identification of different cell types within a heterogeneous population the immune

system [41]. Fluorescent microscopy using acridine orange produces a characteristic chromatin clumping, observed soon after staining. A further refinement is the comet assay, which shows DNA degradation [42].

5.3. PCR method

The innovation of the real-time polymerase chain reaction (PCR) technique played a crucial role in quantification of gene expression, detection of minimal residual disease and pathogen detection [43].

PCR microarray is a relatively new methodology that uses real-time PCR to profile the expression of at least 112 genes involved in apoptosis. These PCR microarrays are designed to determine the expression profile of genes that encode key ligands, receptors, intracellular modulators, and transcription factors involved in the regulation of programmed cell death. This type of assay allows for the evaluation of the expression of a focused panel of genes related to apoptosis and several companies offer apoptosis pathway-specific gene panels [44].

6. CONCLUSION AND RECOMMENDATION

Apoptosis plays important roles both during development and in mature tissues. Multicellular organisms use the physiological mechanisms of cell death to regulate developmental morphogenesis and remove infected, mutated or damaged cells from healthy tissues. It is considered as a potent mechanism of tumor protection, ensuring the selective removal of supernumerary, undesirable or damaged cells. It is also essential in immune response and constitutes an efficient strategy of antiviral defence. Thus apoptosis has been considered as inherent and regulating cellular mechanisms through which there is a control between the production of new cells and individual capacity of the self-destruction of the cells. Apoptosis does not require new transcription or translation, suggesting that the molecular machinery required for cell death lay dormant in the cell, and just requires appropriate activation. The importance of understanding the mechanistic machinery of apoptosis is vital because programmed cell death is being initiated by various physiologic and pathologic stimuli. Moreover, the wide spread involvement of apoptosis in the pathophysiology of disease lends itself to therapeutic intervention at many different checkpoints. Understanding the mechanisms of apoptosis and other variants of programmed cell death at the molecular level provides deeper insight into various disease processes and may thus influence therapeutic strategy.

Based on the above conclusion, the following recommendations are forwarded:

(1) Further study of cellular mechanisms of survival should be developed to design the rational therapeutic agents that can modulate the process of apoptosis in the treatment of degenerative diseases.
(2) Skill should be developed to diagnose different cellular characteristics and their nature of regulation.
(3) Considerable efforts should be made in developing and validating methods to image apoptosis.

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