Course : PG Pathshala-Biophysics
Paper 11 : Cellular and Molecular Biophysics
Module 6 : Apoptosis in Health and Disease
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Introduction:

Apoptosis is a type of programmed cell death common to all multicellular organisms. It is a physiological process responsible for elimination of unwanted, excessive, worn out and damaged cells. It is a highly regulated pathway, which is important for maintenance of homeostasis during embryonic as well as adult life of an organism. Although it is a physiological phenomenon but defects in several components of apoptotic pathway leads to various pathological conditions including abnormal development, neurodegenerative diseases, and carcinogenesis. There are different triggering factors for initiation of apoptosis, which takes place via intrinsic and extrinsic pathways. Role of mitochondrial protein cytochrome 'c' and cysteine proteases called 'caspases' are well defined in mechanism of apoptotic pathways. In this module we will discuss in detail about mechanism of apoptosis and its role in various physiological and pathological conditions.

Objectives:

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1. What is apoptosis?
2. Morphological changes during apoptosis
3. Comparison of apoptosis and necrosis
4. Molecular mechanism of apoptosis
5. Role of apoptosis in physiological and pathological conditions

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1. What is apoptosis?

The word 'Apoptosis' is derived from Greek language and means 'falling off' or 'dropping off' of leaves from a tree or petals from a flower. Apoptosis can be defined as:

'A coordinated and often energy dependent process that involves the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that link the initiating stimuli to the final demise of the cell.'

A great amount of research has been implied to understand the mechanism of programmed cell death (PCD) and apoptosis. The term 'apoptosis' (a-po-toe-sis) was first used in 1972 by Kerr, Wyllie, and Currie to describe a morphologically distinct form of cell death. Horvitz explained the mechanism of apoptosis in detail by his work on development of the nematode *Caenorhabditis elegans.* All cell types are capable of undergoing apoptosis, a process that removes excessive and defective cells, or cells, which are not anymore needed for an organism; without damaging the surrounding cells. This type of cell death seems to be predetermined developmentally and is thus called as programmed cell death (PCD). Normal cells turn apoptotic when they are in excess, have served their function, diseased, become senescent or are a threat to the developing organism. Apoptosis occurs normally during development and aging and also as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defence

mechanism and eliminates cells which are damaged by disease or noxious agents. There are varieties of physiological or pathological stimuli, which can trigger the process of apoptosis. One important feature of apoptosis is that it does not affect the surrounding cells and this process does not provoke an inflammatory response.

2. Morphological changes during apoptosis

During apoptosis, cells break up into fragments, called apoptotic bodies containing parts of cytoplasm and nucleus. The plasma membrane of the apoptotic cell and bodies remains intact, but its structure is altered in such a way that these become "tasty" targets for the phagocytes. The dead cell and its fragments are rapidly consumed, before the contents have leaked out, and therefore cell death by this pathway does not cause an inflammatory reaction in the host. The following morphological features characterize the apoptosis:

2.1 Cell shrinkage: During early phases of apoptosis, cell is smaller in size; the cytoplasm is dense; and the organelles are more tightly packed.

2.2 Chromatin condensation (pyknosis): This is the most distinguishing feature of apoptosis. The chromatin aggregates peripherally, under the nuclear membrane, into dense masses of various shapes and sizes. The nucleus itself may break up, producing two or more fragments (karyorrhexis).

2.3 Formation of cytoplasmic blebs and apoptotic bodies: The apoptotic cell first shows widespread surface blebbing, then undergoes fragmentation into membrane-bound apoptotic bodies containing cytoplasm and tightly packed organelles, with or without nuclear fragments.

2.4 Phagocytosis of apoptotic cells or cell bodies, usually by macrophages: The apoptotic bodies are promptly ingested by phagocytes and degraded within phagolysosomes. Macrophages that engulf and digest apoptotic cells are called "tingible body macrophages." The tingible bodies are the bits of nuclear debris from the apoptotic cells.

On histologic examination, in tissues stained with hematoxylin and eosin, the apoptotic cell appears as a round or oval mass of intensely eosinophilic cytoplasm with fragments of dense nuclear chromatin.

3. Comparison of apoptosis and necrosis

Necrosis is the type of cell death that is associated with loss of membrane integrity and leakage of cellular contents leading to dissolution of cells, largely resulting from the degradative action of enzymes on lethally injured cells. The leaked cellular contents often elicit inflammation, which attempts to eliminate the dead cells and start the subsequent repair process. The enzymes responsible for digestion of the cell may be derived from the lysosomes of the dying cells themselves and from the lysosomes of leukocytes that are recruited as part of the inflammatory reaction to the dead cells. Following are the distinguishing features of apoptosis and necrosis:

Feature	Necrosis	Apoptosis
Cell size	Enlarged (swelling)	Reduced (shrinkage)
Nucleus	Pyknosis → karyorrhexis → karyolysis	Fragmentation into nucleosome size fragments
Plasma membrane	Disrupted	Intact; altered structure, especially orientation of lipids
Cellular contents	Enzymatic digestion; may leak out of cell	Intact; may be released in apoptotic bodies
Adjacent inflammation	Frequent	No
Physiologic or pathologic role	Invariably pathologic (culmination of irreversible cell injury)	Often physiologic; means of eliminating unwanted cells; may be pathologic after some forms of cell injury, especially DNA and protein damage

4. Molecular mechanism of apoptosis

Apoptosis is initiated by various external and internal stimuli leading to activation of molecular pathways, which are highly complex and energy dependent cascade of molecular events controlled by regulated expression of apoptosis related genes and proteins. Before going into the details of molecular pathways of apoptosis, we will discuss some distinct biochemical changes occurring during apoptosis.

4.1 Biochemical changes during apoptosis

The distinctive morphological features of apoptotic cells described above, result from certain characteristic biochemical alterations such as protein cleavage, protein cross-linking, DNA breakdown and phagocytic recognition.

4.1.1 Activation of caspases

Activation of several members of a family of cysteine proteases is a specific feature of apoptosis. They are called as 'caspases', where 'c' refers to presence of cysteine at their active site and 'aspases' signifies their unique ability to cleave after aspartic acid residues. Caspases can be categorized into initiator (2,8,9,10), executioner (3,6,7) and inflammatory (1,4,5) caspases. They function as homodimers, with one domain of each stabilizing the active site of the other. The major effecror caspase in *C.elegans* is CED-3, whereas humans have 15 different caspases. All caspases are present in inactive form as procaspases which must be cleaved to become active. In vertebrates, initiator caspases are activated by dimerization after binding to certain other types of proteins (e.g., Apaf-1), which help the initiators to aggregate. Activated initiator caspases cleave effector caspases (e.g., caspase 3) and activate them. In this way the proteolytic activity of the few initiator caspases becomes rapidly and hugely increased by activation of the effector caspases, leading to a massive increase in the total caspase activity level in the cell and eventually cell death. The various effector caspases differ in the preffered target sequences and cleave proteins of nuclear lamina and cytoskeleton whose cleavage leads to demise of the cell.

4.1.2 DNA and protein breakdown

Apoptotic cells exhibit a characteristic fragmentation of DNA into 180- to 200- base pairs resulting from action of Ca^{+2} and Mg^{+2} dependent endonucleases. A typical "DNA ladder" can be visualized by agarose gel electrophoresis with an ethidium bromide stain and ultraviolet illumination. Extensive protein cross-linking results from expression and activation of tissue transglutaminase.

4.1.3 Membrane alterations and recognition by phagocytes

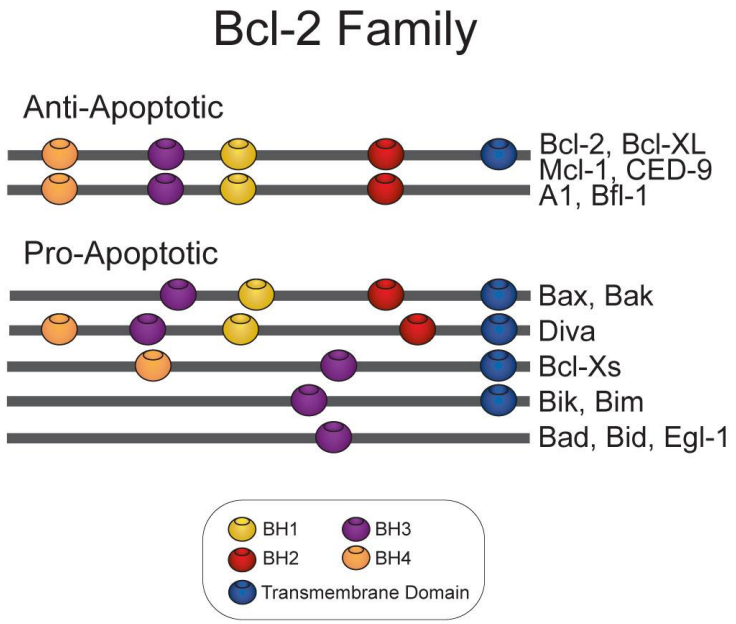
Apoptotic cells display certain cell surface markers which facilitates early recognition by phagocytes, limiting the damage to surrounding tissue. Most notable alteration is the movement of phosphatidyl serine from inner leaflet of lipid bilayer to the outer leaflet. This molecule is recognized by receptors on surface

of phagocytes. Annexin V is a recombinant phosphatidyl serine-binding protein that interacts strongly and specifically with phosphatidyl serine residues and, is used for detection of apoptosis.

4.2 Bcl-2 family of proteins

Bcl-2, an anti-apoptotic protein was the first member of family to be identified. Bcl-2 family includes several pro- and anti-apoptotic molecules and play major role in molecular mechanism of apoptosis. Members of this family possess evolutionary conserved one to four alpha helical BH (Bcl-2 homology) domains, designated BH1, BH2, BH3 and BH4. They control the apoptotic process by governing mitochondrial outer membrane permeability. In vertebrates, anti-apoptotic Bcl-2 resides in the outer mitochondrial membrane and primarily functions to maintain its low permeability, preventing cytochrome c and other proteins localized in the intermembrane space from leaking out into the cytosol and activating apoptotic caspases.

The BH domains are known to be crucial for function of Bcl-2 family of proteins. The anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-xL, conserve all four BH domains. The BH domains also serve to subdivide the pro-apoptotic proteins into those with several BH domains (e.g. Bax and Bak) or those proteins that have only the BH3 domain (e.g. Bim, Bid and Bad). All anti-apoptotic proteins contain BH1 and BH2 domains, some of them contain an additional N-terminal BH4 domain (Bcl-2, Bcl-xL and Bcl-w). On the other hand, all pro-apoptotic proteins contain a BH3 domain necessary for dimerization with other proteins of Bcl-2 family and crucial for their killing activity, some of them also contain BH1 and BH2 domains (Bax and Bak). The BH3 domain is also present in some anti-apoptotic protein, such as Bcl-2 or Bcl-xL. Apoptosis is regulated by a careful balance of anti-apoptotic and multiple proapoptotic BH3 only proteins.



4.3 Pathways of apoptosis

All cells contain intrinsic mechanisms that signal death or survival, and apoptosis results from an imbalance in these signals. The basic mechanism of apoptosis is evolutionary conserved in all

multicellular organisms and in fact some of the major breakthroughs in understanding the mechanism of apoptosis came from studies in the nematode *C.elegans*, whose development proceeds by a highly reproducible, programmed pattern of cell growth followed by cell death. The process of apoptosis may be divided into an initiation phase, during which some caspases become catalytically active, and an execution phase, during which other caspases trigger the degradation of critical cellular components. Initiation of apoptosis occurs principally by signals from two distinct pathways: the intrinsic, or mitochondrial, pathway, and the extrinsic, or death receptor–initiated, pathway. These pathways are induced by distinct stimuli and involve different sets of proteins, although there is some cross-talk between them. Both pathways converge to activate caspases, which are the actual mediators of cell death.

4.3.1 Intrinsic (mitochondrial) pathway of apoptosis

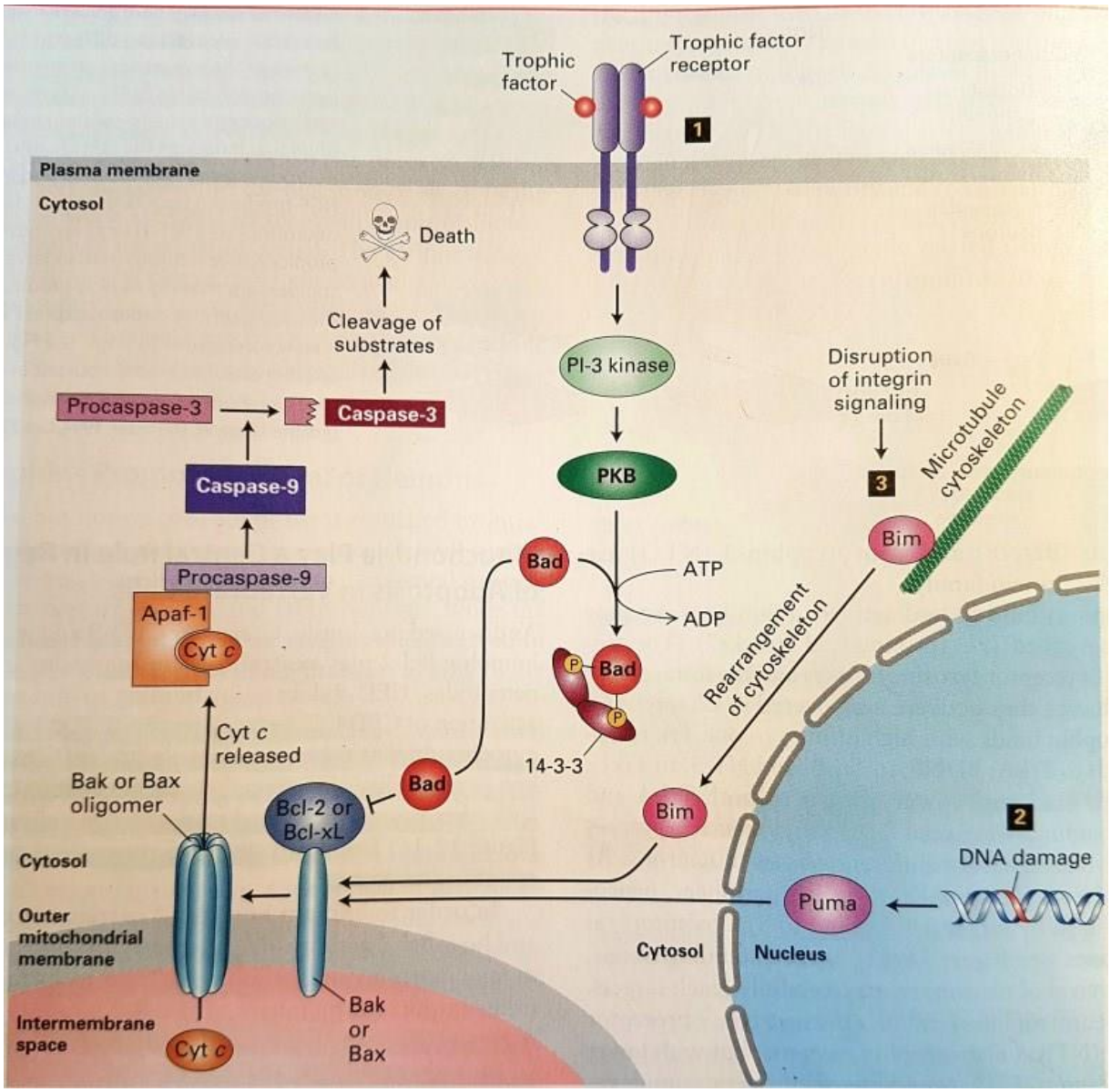
Intrinsic pathway of apoptosis is initiated by intracellular signals produced by a diverse array of non-receptor mediated stimuli. These signals act directly on targets within the cell and mitochondria plays the central role in this pathway. The signals can act in a positive or negative manner. Negative signals include the absence of certain pro-survival growth factors, hormones and cytokines. Examples of stimuli acting in a positive fashion include radiations, toxins, hypoxia, hyperthermia, viral infections and free radicals etc.

Mitochondria plays the central role in intrinsic pathway and apoptotic mitochondrial events are regulated by Bcl-2 family of proteins. In vertebrate cells, Bax and Bak, the two pro-apototic proteins are required for mitochondrial damage and induction of apoptosis. Normally Bax and Bak reside in the outer mitochondrial membrane, tightly bound to Bcl-2. Bax and Bak can be released from Bcl-2 either by being present in excess, by being displaced by binding of certain BH-3 only proteins to Bcl-2, or by direct binding to other BH-3 only proteins. Once released from Bcl-2, they form oligomers and generate pores in the outer mitochondrial membrane. This allows the mitochondrial proteins like cytochrome c, which normally resides in the intermembranous space of mitochondria, to be released into the cytosol. Bax and Bad oligomers, but not Bcl-2 homodimers or Bcl-2/Bax heterodimers, allows influx of ions through the outer mitochondrial membrane, triggering the release of cytochrome c.

When cytochrome c is not present in cytosol, monomeric Apaf-1 remains bound to dATP. Cytochrome c binding with Apaf-1 (Apoptotic protease activating factor 1) leads to cleavage of dATP into dADP and results in dramatic assembly events to form a heptameric wheel of death called as 'apoptosome.' Apoptosome binding activates the initiator caspase-9 by inducing its dimerization. Caspase-9 then leads activation of multiple molecules of effector caspases (e.g, caspase-3) resulting in destruction of critical cellular proteins and cell death. Assembly of Bak/Bax proteins also leads to release of SMAC/DIABLOs (second mitochondria-derived activator of caspases/ direct inhibitor of apoptosis binding protein with low PI) family of proteins from mitochondria. This family of proteins binds to and inhibit IAPs (inhibitor of apoptosis proteins). IAPs normally bind to caspases and keeps them in inactive state. By relieving IAP mediated inhibition, SMAC/DIABLOs promote caspase activity and apoptosis.

The presence of specific trophic factors (e.g., NGF for neurons) leads to phosphorylation of proapoptotic BH3 only protein Bad through receptor tyrosine kinase (TrkA) pathway. Phosphorylated Bad remains complexed to phosphoserine binding protein 14-3-3 in the cytosol. Absence of trophic factors results in unphosphorylated Bad which binds to antiapoptotic Bcl-2 or Bcl-xL on outer mitochondrial membrane. This binding of unphosphorylated Bad inhibits the ability of Bcl-2 and Bcl-xL to bind Bax and Bak, permitting Bak and Bax channels to form and release of cytochrome c and other proteins resulting in cell death. Puma and Noxa are also proapototic BH3 only proteins which are transcriptionally induced by p53 protein responsible for cell death in case of an irreparable damage to DNA. Both Puma and Noxa apparently bind directly to Bax and Bak releasing them from Bcl-2 and permitting formation of mitochondrial pore and apoptosis. Removal of cell from its substratum disrupts integrin signaling, leading

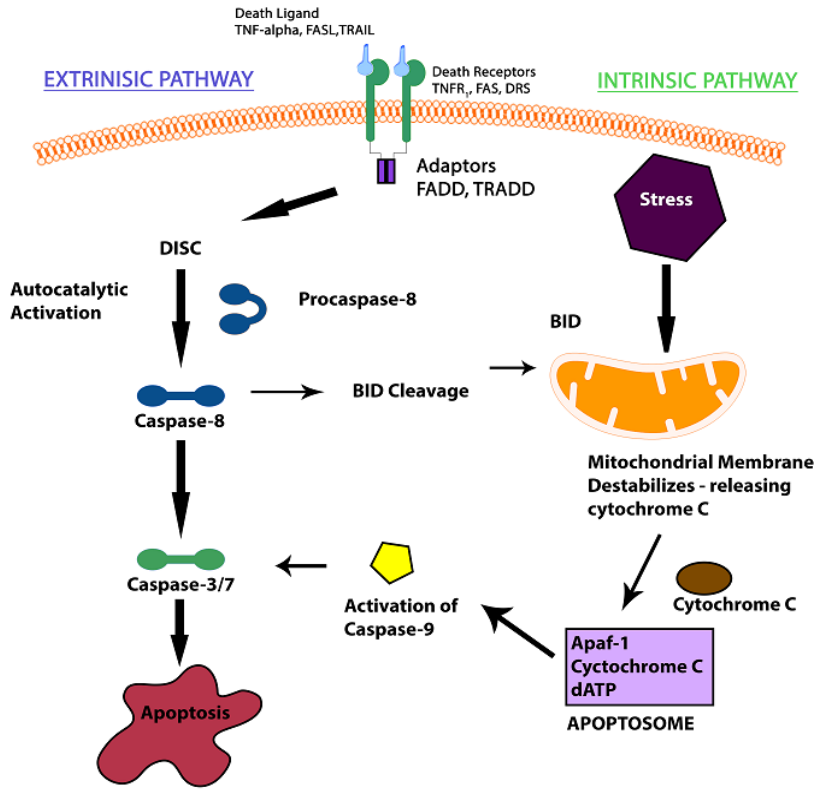
to release of the BH3 only Bim protein from the cytoskeleton. Bim binds to Bak and Bax and leads to cell death by activating pore formation by them.



4.3.2 Extrinsic (death receptor) pathway of apoptosis

Extrinsic pathway of apoptosis is initiated by engagement of death receptors present on plasma membrane of a variety of cells. Death receptors are members of TNF receptor family that contain a cytoplasmic domain involved in protein-protein interactions that is called the 'death domain' because it is essential for delivering death signals from cell surface to intracellular signalling pathways. The best characterized ligands and corresponding death receptors include FasL/FasR and TNFα/TNFR1. Fas ligand is a cell surface protein produced by activated NK cell and cytotoxic T lymphocytes. This signal can trigger death

of certain virus infected, tumor and foreign graft cells. TNFα is released by macrophages and triggers cell death tissue destruction seen in some chronic inflammatory diseases.



Both TNFα and FasL (CD95) are proteins present on the surface of one cell that bind to death receptors on an adjacent cell. Death receptors have a single transmembrane domain and, are activated when binding of an oligomeric ligand brings three receptor molecules into close proximity. The binding of Fas ligand to Fas receptor leads to binding of adapter protein FADD (Fas associated death domain) and binding of TNFα to TNF receptor results in binding of adapter protein TRADD (TNF receptor associated death domain) with recruitment of FADD. FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing silencing complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8. Once activated, caspase-8 activates several effector caspases and the amplification cascade begins.

Caspase-8 also cleaves the BH3 only protein BID. The resulting t-BID fragment then binds to Bcl-2 on the outer mitochondrial membrane, leading to formation of a Bak/Bax channel, release of cytochrome c into the cytosol, and activation of the intrinsic apoptotic pathway as well.

Extrinsic apoptotic pathway can be inhibited by a protein called called Cellular FLICE (FADD-like IL-1β-converting enzyme) -inhibitory protein (c-FLIP) which binds to FADD and caspase -8, rendering them ineffective. Toso is another protein which has been shown to block Fas-induced apoptosis in T cells via inhibition of caspase-8 processing.

4.3.3 Execution pathway and phogocytosis

Both intrinsic and extrinsic pathway of apoptosis converge at the point of execution phase which is marked by activation and actions of effector or executioner caspases (e.g., 3,6,7). These caspases activate cytoplasmic endonucleases, which digests the nuclear material and proteases responsible for degradation of nuclear and cytoskeletal proteins. Activity of effector caspases results in characteristic morphological and biochemical changes seen in apoptotic cells. Caspase-3 cleaves ICAD (inhibitor of caspase activated DNAse) to release endonuclease CAD (caspase activated DNAse) which degrades chromosomal DNA and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies.

The formation of apoptotic bodies breaks the cells up into bite sized fragments which become tasty targets for phagocytes. Apoptotic cells and apoptotic bodies undergo several modifications in their membrane which promotes their rapid clearance by phagocytes before they release their contents (which can result in inflammation). Phospholipid phosphatidyl serine is normally present on inner leaftlet of plasma membrane in healthy cells. This phospholipid flips out in apoptotic cells and, is responsible for recognition by several receptors present on the surface of phagocytes. Cells that are dying by apoptosis secrete soluble factors that recruit phagocytes. Thrombospondin, an adhesive glycoprotein is expressed by some apoptotic bodies, which is recognized by phagocytes. Apoptotic bodies may also become coated with natural antibodies and proteins of the complement system, particularly C1q, which are recognizable by phagocytic cells. Thus, numerous receptors on phagocytes and ligands induced on apoptotic cells are involved in the binding and engulfment of dying cells. This process of phagocytosis of apoptotic cells is so efficient that dead cells disappear, often within minutes, without leaving a trace, and inflammation is absent even in the face of extensive apoptosis.

5. Role of apoptosis in physiological and pathological conditions

5.1 Apoptosis in physiological conditions

Cell death by apoptosis is a normal physiological phenomenon and plays important role in eliminating cells that are no longer needed and maintaining a constant number of cells in various tissues. Physiological role of apoptosis can be summarized in following points:

- The programmed destruction of cells during embryogenesis, including implantation, organogenesis, developmental involution, and metamorphosis. The term “programmed cell death” was originally coined to denote death of specific cell types at defined times during the development of an organism. Apoptosis is a generic term for this pattern of cell death.
- Involution of hormone-dependent tissues upon hormone withdrawal, such as endometrial cell breakdown during the menstrual cycle, ovarian follicular atresia in menopause, the regression of the lactating breast after weaning, and prostatic atrophy after castration.
- Cell loss in proliferating cell populations, such as immature lymphocytes in the bone marrow and thymus that fail to express useful antigen receptors, B lymphocytes in germinal centres, and epithelial cells in intestinal crypts, so as to maintain a steady number or maintenance of homeostasis.
- Eradication of potentially harmful self-reactive lymphocytes, either before or after they have completed their maturation, so as to prevent reactions against one's own tissues.
- Death of host cells that have served their useful purpose, such as neutrophils in an acute inflammatory response, and lymphocytes at the end of an immune response. In these situations cells undergo apoptosis because they are deprived of necessary survival signals, such as growth factors.

- Apoptosis is also necessary to rid the body of pathogen-invaded cells and is a vital component of wound healing in that it is involved in the removal of inflammatory cells and the evolution of granulation tissue into scar tissue. Dysregulation of apoptosis during wound healing can lead to pathologic forms of healing such as excessive scarring and fibrosis.

5.2 Apoptosis in pathological conditions

Defects in the regulation of apoptosis can be a significant components of certain diseases notably, cancer, AIDS, autoimmune lymphoproliferative syndromes, ischemia and neurodegenerative diseases like, Parkinson's disease, Alzheimer's disease, Huntington's disease and Amyotrophic Lateral sclerosis (ALS). Some of these conditions can feature insufficient apoptosis while others may show excessive apoptosis.

- Suppression of apoptosis is thought to play a major role in progression and development of certain cancers. Tumor cells can be resistant to apoptosis by overexpression of antiapoptotic proteins like Bcl-2 or downregulation of proapoptotic proteins like Bax. Both Bcl-2 and Bax expression is regulated by tumor suppressor p53, which is most common mutation noted in human cancers. Certain forms of human B cell lymphoma overexpress Bcl-2. Cytotoxic T cells and NK cells have the function to destroy tumor cells by perforin/granzyme B pathway or death receptor pathway. Defects in the expression of Fas and FasL leads to evasion of apoptosis by certain tumor cells, a feature noted in certain cancers.
- Insufficient apoptosis can also result in conditions like autoimmune lymphoproliferative syndrome (ALPS) where, insufficient apoptosis of autoreactive T cells results in multiple autoimmune diseases. The common conditions associated with ALPS include; hemolytic anemia, immune mediated thrombocytopenia, and autoimmune neutropenia.
- Excessive apoptosis is associated with certain diseases involving autoimmune and neurodegenerative conditions. Human immunodeficiency virus (HIV) infects CD4 T cells and internalized where HIV tat protein is thought to increase the expression of Fas receptor leading to excessive apoptosis of CD4 T cells and ultimately AIDS.
- Improperly folded proteins may arise because of mutations in the genes encoding these proteins or because of extrinsic factors, such as damage caused by free radicals. Excessive accumulation of these proteins in the ER leads to a condition called ER stress, which culminates in apoptotic cell death. Apoptosis caused by the accumulation of misfolded proteins has been invoked as the basis of several degenerative diseases of the central nervous system and other organs. Cell death as a result of protein misfolding is now recognized as a feature of a number of neurodegenerative diseases, including Alzheimer, Huntington, and Parkinson diseases, and possibly type 2 diabetes. Deprivation of glucose and oxygen and stresses such as infections also result in protein misfolding, culminating in cell injury and death.
- Pathologic atrophy in parenchymal organs after duct obstruction, such as occurs in the pancreas, parotid gland, and kidney.

SUMMARY:

1. Apoptosis is a type of programmed cell death, a normal physiological phenomenon to get rid of excessive cells which are either defective or served their function and no longer needed in multicellular organisms.

2. Apoptotic cells feature distinct morphological and biochemical modifications without eliciting inflammation.

3. Apoptosis and necrosis are two distinct modes of cellular killing. Necrosis is invariably a pathological phenomenon with inflammation during the cell death.

4. Caspases are cysteine proteases, which are major enzymes responsible for digestion of various cellular components during apoptosis. Caspases are classified into initiator (2,8,9) and executioner or effector (3,6,7) caspases.

5. Bcl-2 family of proteins play central role in regulating apoptotic process with their antiapoptotic (e.g., Bcl-2, Bcl-xL) and proapoptotic members (e.g., Bax,Bad).

6. Intrinsic or mitochondrial pathway of apoptosis is initiated by intracellular signals and involves release of mitochondrial cyctochrome c and formation of apoptosome leading to activation of caspases and cell death.

7. Extrinsic pathway of apoptosis is initiated by extracellular signals and involves engagement of death receptor by ligands and pathway leading to cell death.

8. Both intrinsic and extrinsic pathways of apoptosis converge at the point of activation of caspases and cellular components digestion by them.

9. Apoptosis is common during developmental phase of organisms to get rid of extra cells and maintenance of homeostasis in various tissues.

10. Excessive or insufficient apoptosis can lead to various pathological conditions including cancers, neurodegenerative diseases, immunological disorders etc.

End of Module 6

Thank you