

**APOPTOSIS: MOLECULAR MECHANISM AND TARGET IN CANCER THERAPY**

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**ABSTRACT**

Programmed cell death, or apoptosis has in the past two years undoubtedly become more investigated biological process. Apoptosis is fundamentally distinct from other types of cell death based on its incidence, morphology and biochemistry. Particular morphological alterations of apoptotic cells include nuclear condensation and fragmentation, cell shrinkage, dynamic membrane blebbing, and loss of adhesion to extracellular matrices or to neighbors. Deeper understanding of the molecular mechanisms of apoptosis and its defective status opens the gate for a new class of targeted therapeutics.

**KEYWORDS:** Apoptosis, Caspases, Cancer.

**INTRODUCTION**

Apoptosis, or programmed cell death (PCD), is a naturally occurring process of cell 'suicide' that plays a crucial role in the development and maintenance of metazoans by eliminating superfluous or unwanted cells.<sup>[1]</sup> Apoptosis, or type 1 cell death, is a significant terminal pathway for cells of multicellular organisms and causes a diversity of biological events such as proliferation/homeostasis, differentiation, development and elimination of harmful cells as a protective strategy to remove infected cells.<sup>[2]</sup> Apoptosis is fundamentally distinct from other types of cell death based on its incidence, morphology and biochemistry. Particular morphological alterations of apoptotic cells include nuclear condensation and fragmentation, cell shrinkage, dynamic membrane blebbing and loss of adhesion to extracellular matrices or to neighbors. Biochemical alterations consist of chromosomal DNA cleavage into internucleosomal fragments, phosphatidylserine externalization and activation of a family of proteases recognized as the caspases. A minor dysfunction of PCD results in various pathological disorders in humans such as various cancers.<sup>[1]</sup>

Apoptosis and necrosis are two distinct processes of cell death that occur inside tissues. Necrosis is a passive form of cell death, that occurs accidentally mainly under environmental perturbations and pathological conditions

(i.e. hypothermia, hypoxia, infection, toxins and trauma) with uncontrolled release of inflammatory cellular contents. Apoptosis is a controlled programmed cell death which is energy dependent and occurs naturally under normal physiological conditions where cell is an active participant in its own demise (also known as cellular suicide).<sup>[3]</sup> Apoptosis is responsible for the removal of undesired or damaged cells during the developmental process. Caspases are a family of cysteinyl dependent aspartate directed proteases that play an essential role in regulating apoptosis at cellular level, which finally causes cellular demise through a cascade of molecular events.<sup>[4]</sup>

The biochemical basis for apoptotic cell death is constitutively present in virtually all mammalian cells and can be activated by a wide variety of extra and intracellular signals.<sup>[5]</sup> The induction phase of PCD or apoptosis is characterized by an extreme heterogeneity of potential PCD-triggering signal transduction pathways. Although apoptosis research has exponentially grown still some fundamental questions concerning the molecule and biochemical mechanisms of apoptosis remain to be elucidated. A great deal of effort has been dedicated to biochemical studies of its initiation and regulation, in order to develop new methods of enhancement or inhibition of the process in disease, with considerable benefit for patients.<sup>[6]</sup> In contrast, relatively

little is known about the mechanism involved in the execution of apoptosis.<sup>[7]</sup>

### Pathway of Apoptosis

Apoptosis is recognized as the most important form of cell death and its molecular signaling pathway is well known. Identification of apoptotic mechanisms is critical and facilitates the understanding of the pathogenesis of diseases as a result of dysfunctional apoptosis. This, in turn, may assist in developing new drugs that target specific apoptotic pathways or genes. In mammals, there are two central apoptotic pathways: the extrinsic pathway (death receptor mediated pathway) and the intrinsic pathway (mitochondrial mediated pathway).<sup>[8]</sup> Moreover, besides these two pathways, there are additional pathways of caspase activation that are less well known,

including an initiator role of caspase-12 or caspase-2 in apoptosis activated by endoplasmic reticulum stress (ER).<sup>[9]</sup> The perforin/granzyme pathway is another pathway that involves T-cell mediated cytotoxicity. This pathway can induce apoptosis via either granzyme A or granzyme B. All these apoptotic pathways (extrinsic, intrinsic, and granzyme B pathways) converge on the same terminal or execution pathway (Fig 1).<sup>[10]</sup> The key regulatory proteins in both intrinsic and extrinsic pathways are the caspases. Three groups of mammalian caspases exist on the basis of specific functions in different pathways, including developmental, inflammatory and apoptotic pathways. Thus, some caspases, such as caspase-1, do not have a role in apoptosis execution, while several caspases have dual roles both in nonapoptotic and apoptotic signaling.<sup>[11]</sup>

### INDUCTION PHASE

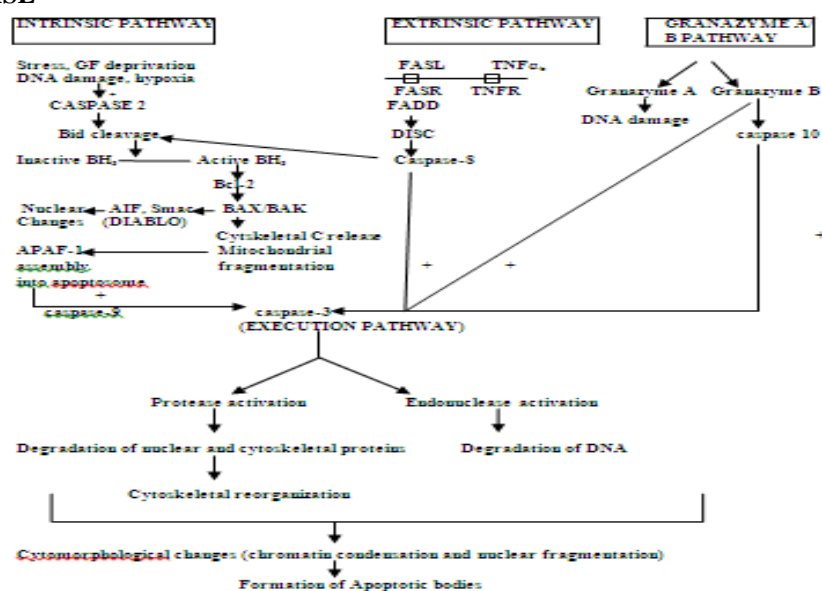


Fig1: Showing Induction and Execution phase of Apoptosis.<sup>[10]</sup>

#### 1. EXTRINSIC PATHWAY

The Extrinsic pathway begins outside a cell, when conditions in the extracellular environment determine that a cell must die. The extrinsic pathway is activated by external signals, such as Fas ligand (FasL) and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ). FasL binds to Fas receptor, a death receptor and Fas - associated death domain (FADD). Binding of FasL to Fas and FADD, activates the formation of death inducing signalling complex protein (DISC), which converts pro caspase-8 to active caspase-8. Active caspase-8 has got two roles, either it activates executioner caspases (caspase-3) or cleave the BH3-only protein (Bid), to cleaved Bid (tBid) and BH3. The tBid then activates the intrinsic apoptotic pathway by triggering oligomerization of the proapoptotic proteins, Bax and Bak.<sup>[4]</sup>

#### 2. Intrinsic Pathway

Injury inside the cell triggers the intrinsic pathway. Intrinsic stresses such as oncogenes, direct DNA damage, hypoxia, and survival factor deprivation activate

this pathway. The active tBid generated from the extrinsic pathway is the connecting link between the extrinsic and the intrinsic pathway. In a different cascade, activated caspase-2 also cleaves Bid to tBid which initiates intrinsic pathway.<sup>[11]</sup> The tBid translocates into the mitochondria, which triggers the activation of Bcl-2 proteins (Bax/Bak). Bax/Bak induces mitochondrial outer membrane permeabilization (MOMP) and release the cytochrome C from intermembrane space of mitochondria into the cytoplasm.<sup>[12]</sup> Inside the cytoplasm, Cytochrome C induces ATP -dependant oligomerization of the adaptor protein Apaf -1 (apoptotic protease activation factor-1), which forms active caspase-9 (through formation of the apoptosome complex) and finally activates caspase -3. Other apoptogenic molecules released from the mitochondria along with cytochrome C, are AIF (apoptosis inducing factor) and Smac (second mitochondria derived activator of caspase, also called DIABLO). AIF directly translocates to the nucleus and triggers caspase - independent nuclear changes.<sup>[4]</sup> Smac,

activates apoptosis by neutralizing the inhibitory activity of IAPs (inhibitory apoptotic proteins) those inhibit caspases.<sup>[13]</sup>

### 3. Additional Pathway

There is an additional pathway that involves T-cell mediated cytotoxicity and perforin - granzyme - dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme a, family of serine proteases.<sup>[4]</sup> Granzyme A pathway causes cell death via single stranded DNA damage which is caspase independent<sup>[14]</sup> where as granzyme B mediated cascade is caspase dependant leading to activation of caspase-3 either directly or through caspase-10.<sup>[2]</sup> Granzyme B also utilizes the mitochondrial pathway for amplification of the death signal by specific cleavage of Bid and induction of cytochrome C release.<sup>[4]</sup>

### EXECUTION PHASE

The extrinsic and intrinsic pathways and perforin pathway all lead to the final pathway of apoptosis. The execution phase includes activation of execution caspases, which are characteristic of the final phase of apoptosis that is associated with the activation of cytoplasmic endonucleases and proteases. This leads to the degradation of nuclear materials and cytoskeletal proteins. Both morphological and biochemical changes characteristic of apoptotic cells are mediated by effector "executioner" caspases such as caspase-3, caspase 6 and caspase-7. Activation of these caspases results in cleavage of various substrates such as cytokeratins, PARP (poly ADP ribose polymerase), plasma membrane cytoskeletal protein alpha fodrin, and nuclear mitotic apparatus protein (NuMA).<sup>[15]</sup>

Caspase-3 is known to be the most important caspase among the executioner caspases and its activation is mediated by initiator caspases such as caspase-8, caspase- 9, or caspase-10.<sup>[15]</sup> In apoptotic cells, activation of caspase-3 mediates activation of various substrates such as endonuclease caspase-activated DNase (CAD), whose activation promotes protein degradation that subsequently causes chromatin condensation in apoptotic cells.<sup>[16]</sup> Additionally, activation of keratin 18 by caspase-3 mediates formation of apoptotic bodies during the course of apoptosis. Moreover, caspase-cleaved gelsolin functions as actin filaments in vitro in a Ca<sup>2+</sup>-independent manner.<sup>[15]</sup> Expression of the gelsolin cleavage product in multiple cell types triggers the cells to become round, detach from the plate and undergo nuclear fragmentation,<sup>[17]</sup> suggesting an important role for activated caspase-3-induced cleavage of gelsolin in apoptosis regulation.<sup>[15]</sup>

After the execution phase is complete, unwanted cellular components should be removed. Therefore, the last step of apoptotic process involves phagocytic uptake of apoptotic cells. Phospholipid asymmetry and externalization of phosphatidylserine on the surface of

apoptotic cells and membrane fragmentation are characteristic of the execution phase. Translocation of phosphatidylserine to the outer side is thought to be mediated by the loss of aminophospholipid translocase activity and nonspecific flip-flop of phospholipids of various classes.<sup>[15,18]</sup> Caspase-independent phosphatidylserine exposure occur during apoptosis of primary T-lymphocytes,<sup>[19]</sup> phosphatidylserine externalization in response to oxidative stress occurs in erythrocytes.<sup>[14]</sup> Moreover, externalization of phosphatidylserine in the execution phase appears to be an essential cellular mechanism for removing apoptotic cells since the appearance of phosphotidylserine on the outer leaflet of apoptotic cells facilitate non-inflammatory phagocytic recognition, thereby allowing their early uptake and finally their removal.<sup>[19]</sup>

### Apoptosis Defect and Cancer

Apoptosis is a cellular procedure that is regulated by different groups of executioner and regulatory molecules and their aberrant function is fundamental to the growth of tumors and the development of anticancer drug resistance. Therefore, apoptosis has become one of the prime molecular targets for drug discovery and development, particularly for diseases like cancer.<sup>[4]</sup>

Defects in programmed cell death (apoptosis) mechanisms play important roles in tumor pathogenesis, allowing neoplastic cells to survive over intended lifespans, subverting the need for exogenous survival factors and providing protection from oxidative stress and hypoxia as the tumormass expands. That gives time for accumulation of genetic alterations that deregulate cell proliferation, interfere with differentiation, promote angiogenesis and increase invasiveness during tumor progression.<sup>[20]</sup> Apoptosis defects are now considered an important complement of protooncogene activation, as many deregulated oncoproteins that drive cell division also trigger apoptosis (e.g., Myc, E1a and Cyclin-D1).<sup>[4]</sup> On the other hand, the noncancerous cells have a DNA repair machinery. Defects in DNA repair and/or chromosome segregation normally trigger cell suicide as a defense mechanism for eradicating genetically unstable cells and thus such suicide mechanism's defects permit survival of genetically unstable cells, providing opportunities for selection of progressively aggressive clones and may promote tumorigenesis.<sup>[21]</sup>

There are varieties of molecular mechanisms that tumor cells use to suppress apoptosis. Tumor cells can acquire resistance to apoptosis by the expression of antiapoptotic proteins such as Bcl-2 or by the downregulation or mutation of proapoptotic proteins such as BAX. Since the expression of both Bcl-2 and BAX is regulated by the p53 tumor suppressor gene,<sup>[22]</sup> some forms of human B-cell lymphoma have Bcl-2 overexpression. That example represents the first and strongest lines of evidence that failure of cell death contributes to cancer. Apoptosis defects may allow epithelial cells to survive in a suspended state, without attachment to extracellular

matrix which facilitate metastasis.<sup>[4]</sup> They also promote resistance to the immune system, including many weapons of cytolytic T cells (CTLs) and natural killer (NK) cells used for attacking tumors that depend on integrity of the apoptosis machinery.<sup>[23]</sup>

#### **Carcinogenesis via Intrinsic signaling defects**

Mitochondria-dependent apoptosis is one of the most important pathways for apoptosis induction. That is why disturbing this pathway is an effective way to inhibit apoptosis. Bcl-2 family proteins are central regulators of the intrinsic pathway, which either suppress or promote changes in mitochondrial membrane permeability required for release of cyt-c and other apoptogenic proteins.<sup>[24]</sup> Antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, Bcl-W, Mcl-1 and Bfl-1/A1), which display sequence homology in all BH1- BH4 domains, promote cell survival, whereas proapoptotic proteins mediate receptor-, mitochondria-, or endoplasmic reticulum (ER) stress dependent apoptosis. The latter group is subdivided into multidomain or BH3-only proteins. The former consists of Bax and Bak, which are essential for apoptosis. The BH3-only proteins are further divided into two subclasses: “activators” (e.g., Bim and tBid), which directly activate Bax/Bak to induce mitochondrial outer membrane permeabilization (MOMP) and “sensitizers/derepressors” (e.g., Bad, Bik, Bmf, Hrk, Noxa, and Puma), which do not activate Bax/Bak directly but instead neutralize antiapoptotic proteins.<sup>[24]</sup> The central role that Bax/Bak play in apoptosis is supported by evidences that BH3-only proteins fail to trigger apoptosis in Bax/Bak-deficient cells.<sup>[24]</sup> Antiapoptotic proteins block death signaling by antagonizing the actions of Bax/Bak through partially known mechanisms. It has been summarized, that antiapoptotic proteins prevent Bax/Bak activation by sequestering/inhibiting “activator” BH3-only proteins and/or directly inhibiting Bax/Bak activation.<sup>[24]</sup> “Sensitizer” BH3-only proteins displace “activator” BH3-only proteins from antiapoptotic proteins, leading to Bax/Bak activation. Alternatively, BH3-only proteins may directly neutralize/inhibit antiapoptotic proteins, releasing their inhibition of Bax/Bak.

Overexpression of antiapoptotic Bcl-2 or Bcl-xL probably occurs in more than half of all cancers,<sup>[24]</sup> rendering tumor cells resistant to myriad apoptotic stimuli, including most cytotoxic anticancer drugs. For example, forced expression of Bcl-2 protein from plasmid vectors, in contrast, abrogates sensitivity to the apoptosis promoting effects of antiestrogens in breast cancer lines, while antisense Bcl-2 prevents estrogen-mediated apoptosis suppression, thus establishing a direct functional connection between ER and Bcl-2 and suppression of apoptosis.<sup>[25]</sup>

#### **Carcinogenesis via Extrinsic Pathway signalling defect**

The extrinsic pathway is activated *in vivo* by TNF family ligands that engage DD-containing receptors, resulting in

activation of DED-containing caspases. The “death ligands” are expressed on CTLs, NK cells and other types of immune relevant cells (activated monocytes/macrophages and dendritic cells) and are used as weapons for eradication of transformed cells.<sup>[24]</sup> Mice-based studies on genetic alterations in genes encoding death ligands or their receptors, as well as use of neutralizing antibodies and Fc-fusion proteins, have provided evidence of important roles in tumor suppression by cellular immune mechanisms. Fas ligand (FasL) is important for CTL-mediated killing of some tumor targets and TRAIL (Apo2 ligand) is critical for NK-mediated tumor suppression. Some tumor cells resist the response of the death receptor pathway to FasL produced by T cells to evade immune destruction. Many tumor cell lines display intrinsic resistance to TRAIL even though they express the necessary cell surface receptors confirming that, during evolution of tumors *in vivo*, selection occurs for malignant clones capable of withstanding immune attack. This could occur in a variety of ways including downregulation of the Fas receptor, expression of nonfunctioning Fas receptor and secretion of high levels of a soluble form of the Fas receptor. That will directly sequester the Fas ligand or expression of Fas ligand on the surface of tumor cells.<sup>[2]</sup> Some tumor cells are capable of a Fas ligand-mediated “counter attack” that results in apoptotic depletion of activated tumor infiltrating lymphocyte.<sup>[24]</sup> Thus, successful biological therapy depends on restoring competency of the extrinsic pathway.

#### **Carcinogenesis via Convergence Pathway Inhibition.**

The intrinsic and extrinsic pathways for caspase activation converge on downstream effectors caspases. Mechanisms for suppressing apoptosis at this distal step and their relevance to cancer are becoming progressively clear. In this regard, the apoptosis-inhibiting proteins (IAPs) represent a family of evolutionarily conserved apoptosis suppressors, many of which function as endogenous inhibitors of caspases. All members of this family, contain at least one copy of a so-called BIR (baculovirus iap repeat) domain, a zinc binding fold, which is important for their antiapoptotic activity, present in 1–3 copies. Caspases-3 and -7, as well as caspase-9 (intrinsic pathway), are directly affected by the human IAP family members, XIAP, cIAP1 and cIAP2. For XIAP, the second BIR domain and the linker region between BIR1 and BIR2 are required for binding and suppressing caspases-3 and -7, while the third BIR domain binds caspase-9. Thus, different domains in the multi-BIR containing IAPs are responsible for suppression of different caspases. IAP family member Livin (ML-IAP) contains a single BIR and inhibits caspase-9 but not caspase-3 and -7. Survivin also contains a single BIR which associates with caspase-9.<sup>[24]</sup>

IAPs are overexpressed in many cancers, while more detailed information is needed about the exact deregulation of the 8 members of this gene family in specific types of cancer. Those IAPs elevations found in

cancers are important for maintaining tumor cell survival and resistance to chemotherapy as confirmed by antisense-mediated reductions experiments. Targeting IAP-family members XIAP, cIAP1, Survivin, or Apollon can induce apoptosis in tumor cell lines in culture or sensitize cells to cytotoxic anticancer drugs.<sup>[28]</sup>

### Molecular Targeting Therapies and apoptosis

Expression of inhibitors of apoptosis as well as inactivation of apoptosis promoters is observed in human cancers.<sup>[24]</sup> Significant advances have been made in discovery and validation of several types of novel cancer therapeutics. In past decade such novel therapeutics tries to prime the apoptotic machinery to act as promising apoptosis-inducing agents, bearing high hopes for the management of cancers resistant to conventional treatments.<sup>[26]</sup> This therapeutics can be used either alone or in combination with some of the conventional therapies. A few of the most promising therapeutic strategies which target the induction of apoptosis have been given below.

1. Targeting Anti apoptotic Bcl-2 Family Members
2. Targeting cell cycle control system for apoptosis induction.
3. Targeting DNA repair system for apoptosis induction.
4. Targeting death receptors.
5. NF-kb as a target for apoptosis induction.
6. Targeting multiple pathway regulators.
7. Targeting heat shock chaperons.

### CONCLUSION

Cancer-associated defects in apoptosis play a role in treatment resistance with conventional therapies like chemotherapy and radiotherapy, increasing the threshold for cell death and thereby requiring higher doses for tumor killing agents. Hence, deeper understanding of the molecular mechanisms of apoptosis and its defective status opens the gate for a new class of targeted therapeutics. The success of each therapeutic strategy depends mainly on the ability of therapeutic tool to induce apoptosis either by targeting over expressed anti-apoptotic proteins or by stimulating expression of pro-apoptotic molecules. Many therapeutic agents are however challenged by the maneuvers from cancer cells to survive treatments. Alterations in the expression levels or mutation in the drugs target may have an impact on apoptosis by such drug. Dysregulated or defective apoptosis regulation is a fundamental aspect of the tumor biology. Successful eradication of cancer cells by non surgical means is ultimately approached via induction of apoptosis. Therefore, all the cancer drug designers try either to activate the inactivated apoptotic mechanism or rectify a defective one. All cytotoxic anticancer therapies currently in clinical use, when they work, induce apoptosis of malignant cells.<sup>[24]</sup>

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