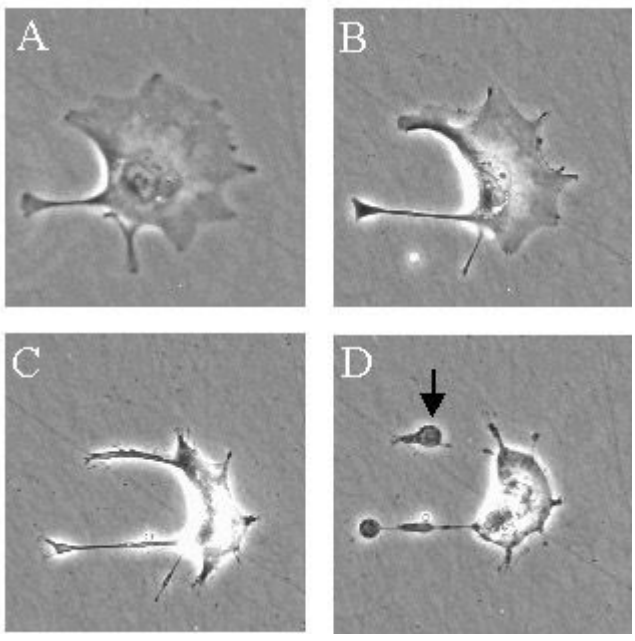
APOPTOSIS

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide).

Upon receiving specific signals instructing the cells to undergo apoptosis a number of distinctive biochemical and morphological changes occur in the cell. A family of proteins known as caspases is typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular substrates that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus. The result of these biochemical changes is appearance of morphological changes in the cell.

Some of these changes are illustrated in Figure 1, which shows time-lapse microscopy images of a trophoblast cell undergoing apoptosis.

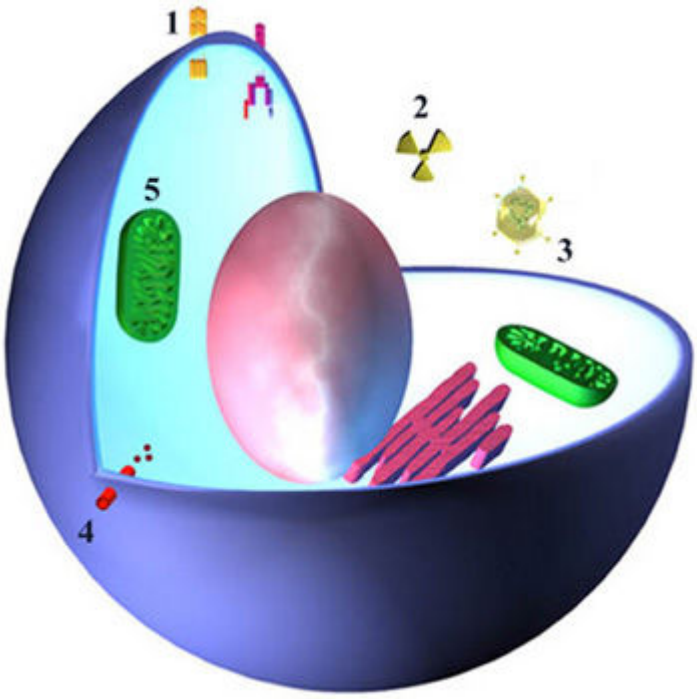


Typically, the cytoplasm begins to shrink following the cleavage of lamins and actin filaments (A). Nuclear condensation can also be observed following the breakdown of chromatin and nuclear structural proteins, and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance (B). Cells continue to shrink (C), packaging themselves into a form that allows for easy clearance by macrophages. These phagocytic cells are responsible for removing apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell

death. In order to promote their phagocytosis by macrophages, apoptotic cells often ungergo plasma membrane changes that trigger the macrophage response. One such change is the translocation of phosphatidylserine from the inner leaflet of the cell to the outer surface. Membrane changes can often be observed morphologically through the appearance of membrane blebs (D) or blisters which often appear towards the end of the apoptotic process. Small vesicles called apoptotic bodies are also sometimes observed (D, arrow).

Induction of apoptosis

There are a number of mechanisms through which apoptosis can be induced in cells. The sensitivity of cells to any of thses stimuli can vary depending on a number of factors such as the expression of pro- and anti-apoptotic proteins (eg. the Bcl-2 proteins or the Inhibitor of Apoptosis Proteins), the severity of the stimulus and the stage of the cell cycle. Some of the major stimuli that can induce apoptosis are outlined in the illustration below.



In some cases the apoptotic stimuli comprise extrinsic signals such as the binding of death inducing ligands to cell surface receptors **(1)** or the induction of apoptosis by cytotoxic T-lymphocytes by granzyme **(4)**. The latter occurs when T-cells recognise damaged or virus infected cells and initiate apoptosis in order to prevent damaged cells from becoming neoplastic (cancerous) or virus-infected cells from spreading the infection.

In other cases apoptosis is initiated following intrinsic signals that are produced following cellular stress. Cellular stress may occur from exposure to radiation **(2)** or chemicals or to viral infection **(3)**. It might also be a consequence of growth factor deprivation or oxidative stress. In general intrinsic signals initiate apoptosis via the

involvement of the mitochondria **(5)**. The relative ratios of the various bcl-2 proteins can often determine how much cellular stress is necessary to induce apoptosis.

Death receptors

Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific ligands. They play an important role in apoptosis and can activate a caspase cascade within seconds of ligand binding. Induction of apoptosis via this mechanism is therefore very rapid. Death receptors belong to the tumour necrosis factor (TNF) gene superfamily and generally can have several functions other than initiating apoptosis. The best characterised of the death receptors are CD95 (or Fas), TNFR1 (TNF receptor-1) and the TRAIL (TNF-related apoptosis inducing ligand) receptors DR4 and DR5.

Signaling by Tumour Necrosis Factor Receptor-1 (TNFR1)

TNF is produced by T-cells and activated macrophages in response to infection. By ligating TNFR1, TNF can have several effects (see Figure 1). In some cells it leads to activation of NF-kB and AP-1 which leads to the induction of a number of proinflammatory and immunomodulatory genes. In some cells, however, TNF can also induce apoptosis, although receptor ligation is rarely enough on its own to initiate apoptosis as is the case with CD95 ligand binding.

Binding of TNF alpha to TNFR1 results in receptor trimerisation and clustering of intracellular death domains. This allows binding of an intracellular adapter molecule called TRADD (TNFR-associated death domain) via interactions between death domains. TRADD has the ability to recruit a number of different proteins to the activated receptor. Recruitment of TRAF2 (TNF-associated factor 2) leads to activation of NF-kB and the JNK/Ap-1 pathway.

TRADD can also associate with FADD, which leads to the induction of apoptosis via the recruitment and cleavage of pro-caspase 8. TNFR1 is also able to mediate apoptosis through the recruitment of an adapter molecule called RAIDD (RIP-associated ICH-1 / CED-3 homologous protein with a death domain). RAIDD associates with RIP through interactions between death domains and can recruit caspase 2 through an interaction with a motif, similar to the death effector domain, known as CARD (caspase recruitment domain). Recruitment of caspase 2 leads to induction of apoptosis.

Signaling by CD95 / Fas

There are three main roles of CD95:

1. Cytotoxic T-cell mediated killing of cells (for example, CTL-mediated killing of virus-infected cells)
2. Deletion of activated T-cells at the end of an immune response
3. Destruction of inflammatory and immune cells in immune-privileged sites

The activation of apoptosis through CD95/Fas signaling is shown in Figure 2. The ligand for CD95 (CD95L or FasL) is a trimer that on association with the receptor promotes receptor trimerisation that in turns results in intracellular clustering of parts of the receptor called death domains (DD). This allows an adapter protein called FADD (Fas-associated death domain) to associated with the receptor through an interaction between homologous death domains on the receptor and on FADD. As well as containing a death domain, FADD also contains a death effector domain (DED). The death effector domain allows binding of pro-caspase 8 to the CD95-FADD complex. Pro-caspase 8 (also known as FLICE) associates with FADD though its own death effector domain, and upon recruitment by FADD is immediately cleaved to produce caspase 8. This then triggers activation of execution caspases such as caspase 9. The complex of proteins – CD95, FADD and pro-caspase 8 – that trigger apoptosis is also known as DISC or Death Inducing Signaling Complex.

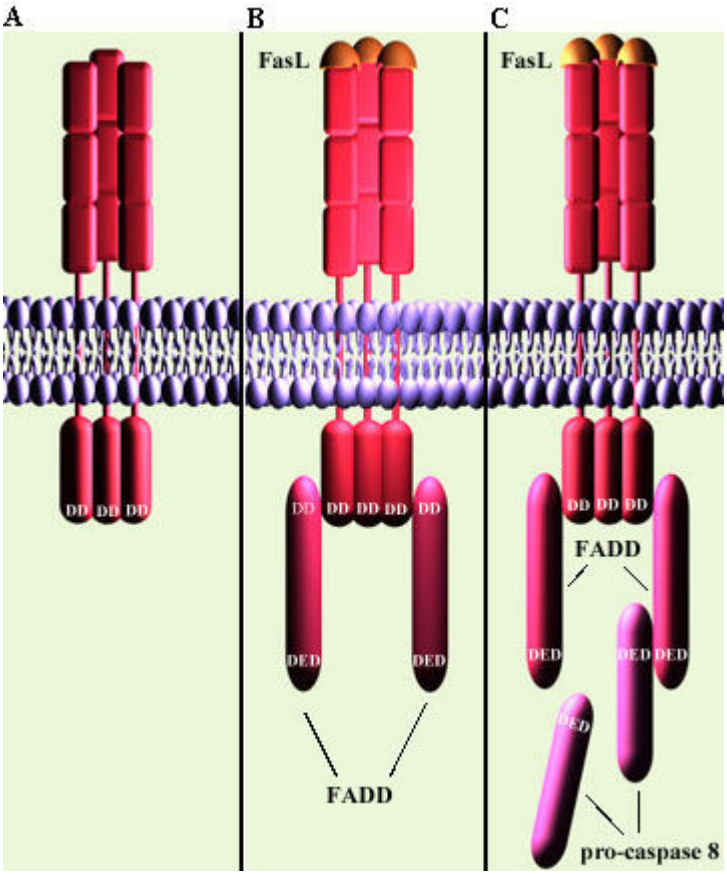


Figure 1: Activation of apoptosis through CD95 / Fas

Induction of apoptosis by TRAIL

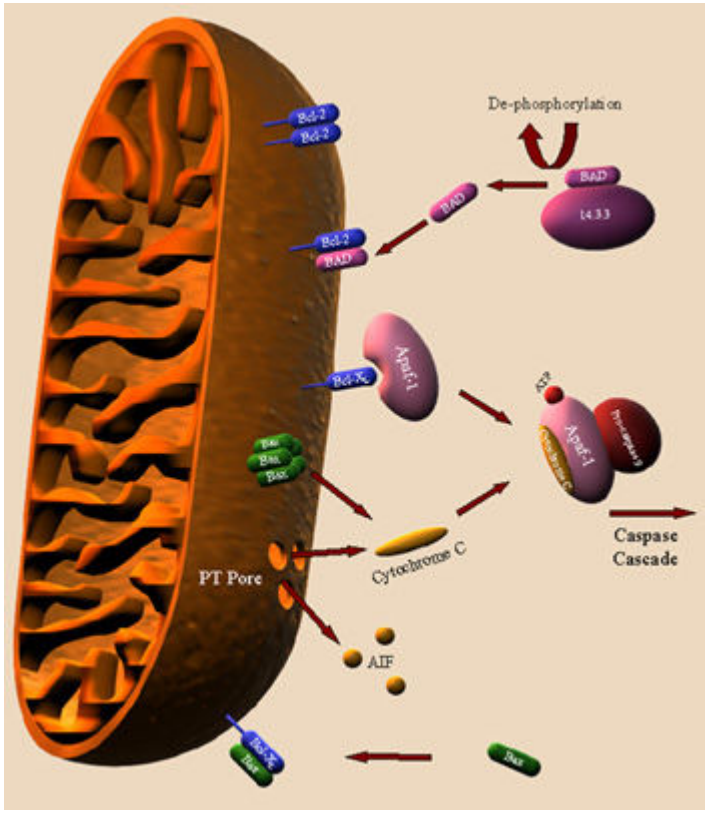
In a number of ways TRAIL (TNF-related apoptosis inducing ligand) is similar in action to CD95. Binding of TRAIL to its receptors DR4 or DR5 triggers rapid apoptosis in many cells, however unlike CD95, its expression has been shown to be constitutive in many tissues. The DR4 and DR5 receptors contain death domains in their intracellular domain, but as yet no adapter molecule (such as FADD or TRADD) has been identified that associates with the receptor to initiate apoptosis. Work in

FADD-deficient mice has indicated that FADD is not essential for triggering apoptosis via these receptors.

Since DR4 and DR5 mRNA has been shown to be expressed constitutively in several tissues, it has been suggested that there are mechanisms that protect cells from apoptosis. One possible mechanism of protection is based on a set of decoy receptors that compete for binding of TRAIL with the DR4 and DR5 receptors. The decoy receptors are called DcR1 and DcR2. Both of these receptors are capable of competing with DR4 or DR5 receptors for binding to the ligand (TRAIL), however ligation of these receptors does not initiate apoptosis since DcR1 does not possess a cytoplasmic domain, while DcR2 has a truncated death domain lacking 4 out of 6 amino acids essential for recruiting adapter proteins.

Role of Mitochondria

Mitochondria play an important role in the regulation of cell death. For example, anti-apoptotic members of the Bcl-2 family of proteins, such as Bcl-2 and Bcl-XL, are located in the outer mitochondrial membrane and act to promote cell survival. Many of the pro-apoptotic members of the Bcl-2 family, such as Bad and Bax also mediate their effects though the mitochondria, either by interacting with Bcl-2 and Bcl-XL, or through direct interactions with the mitochondrial membrane. The roles that mitochondria play in apoptosis is summarised in the illustration below. Information on each of these roles is given below.



Mitochondria have the ability to promote apoptosis through release of cytochrome C, which together with Apaf-1 and ATP forms a complex with pro-caspase 9, leading to activation of caspase 9 and the caspase cascade.

It is unknown what factors promote the release of cytochrome C from the mitochondria. Since Bax, and other Bcl-2 proteins, show structural similarities with pore-forming proteins. It has therefore been suggested that Bax can form a transmembrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and efflux of cytochrome C and AIF (apoptosis inducing factor). It is thought that Bcl-2 and Bcl-XL act to prevent this pore formation. Heterodimerisation of Bax or Bad with Bcl-2 or Bcl-XL is thought to inhibit their protective effects. It is also thought that proteins such as Bax and Bad can promote the formation of the large diameter PT pore, with subsequent loss of cytochrome C and initiation of apoptosis.

Caspases

Caspases are a family of proteins that are one of the main effectors of apoptosis.The caspases are a group of cysteine proteases that exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis.

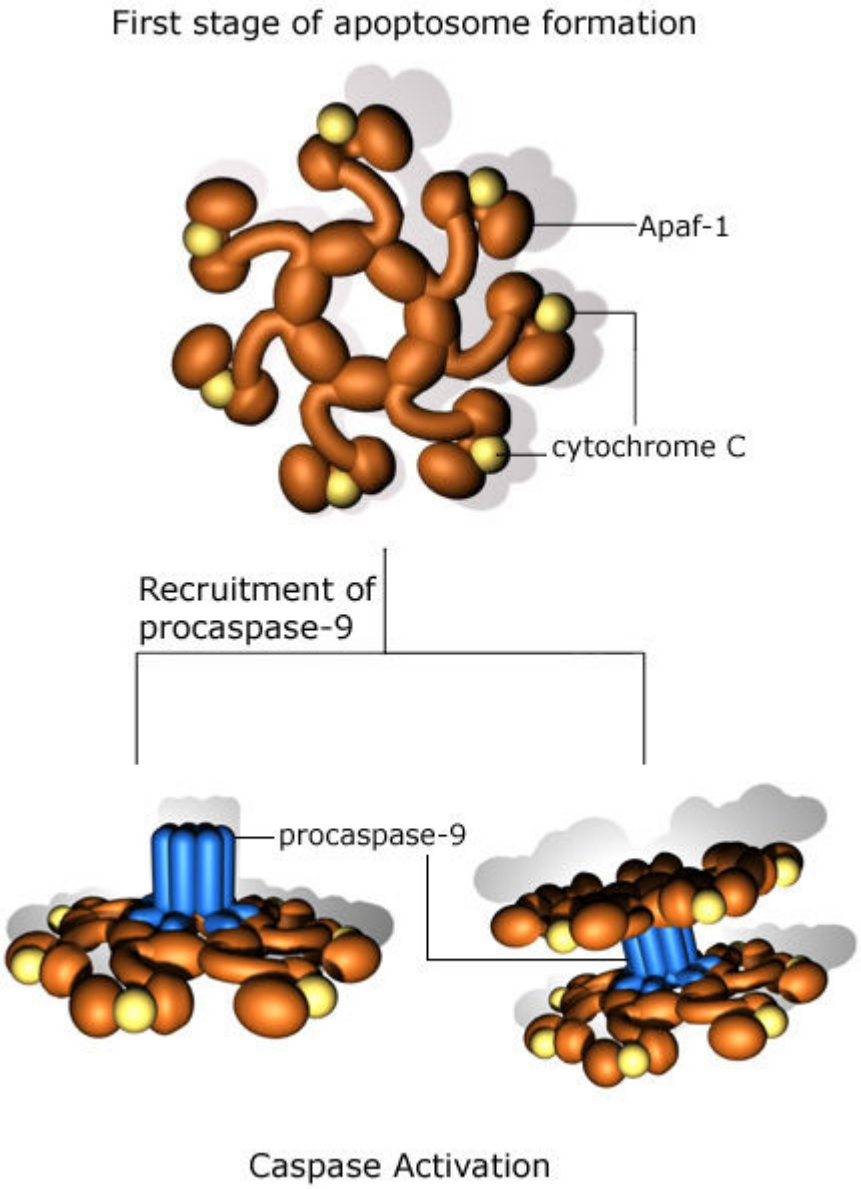
Induction of apoptosis via death receptors results in the activation of an initiator caspase such as caspase 8 or caspase 10. These caspases can then activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6. These caspases are responsible for the cleavage of the key cellular proteins that leads to the typical morphological changes observed in cells undergoing apoptosis. A list of the main caspases and some of their substrates is shown in the table below.

Caspase	Alternate name	Substrates	Function (and notes)
Caspase-1	ICE	pre-Interleukin-1b Interleukin-18 Lamins	Processing of interleukins (inflammation). Can also induce apoptosis depending on isoform and if overexpressed.
Caspase-2	Ich-1 (human), Nedd2 (rat, mouse)	Golgin-160 Lamins (?)	Apoptosis (activity supressed by serum deprivation)
Caspase-3	CPP32, Yama, apopain	PARP SREBs Gelsolin Caspase-6 Caspase-7 Caspase-9 DNA-PK MDM2 Gas2 Fodrin	Apoptosis

		b-Catenin Lamins NuMA HnRNP proteins Topoisomerase I FAK Calpastatin $p21^{Waf1}$ Presenelin2 ICAD	
Caspase-4	Ich-2, $ICE_{rel}II$	Caspase-1	Inflammation/Apoptosis (note: this could be the human form of mouse caspase-11). Related to human caspase-5 and caspase-1
Caspase-5	$ICE_{rel}III$, TY	?	Inflammation/Apoptosis (related to human caspase-4 and caspase-1)
Caspase-6	Mch2	PARP Lamins NuMA FAK Caspase-3 Keratin-18	Apoptosis
Caspase-7	Mch3, ICE-LAP3, CMH-1	PARP Gas2 SREB1 EMAP II FAK Calpastatin $p21^{Waf1}$	Apoptosis (activity blocked by cIAP1 and cIAP2) Similar in structure and substrate specificity to caspase-3
Caspase-8	FLICE, MACH, Mch5	Caspase-3 Caspase-4 Caspase-6 Caspase-7 Caspase-9 Caspase-10 Caspase-13 PARP Bid	Apoptosis (death receptors)
Caspase-9	Apaf-3, ICE-LAP6, Mch6	Caspase-3 pro-Caspase-9 Caspase-7 PARP	Apoptosis
Caspase-10	FLICE-2, Mch4	Caspase-3 Caspase-4 Caspase-6 Caspase-7 Caspase-8 Caspase-9	Apoptosis (death receptors)
Caspase-11	Ich-3, ICE_B	?	Murine caspase similar to human

			caspase-4. Belongs to the same family as caspase-3 of enzymes. May be involved in inflammation and apoptosis
Caspase-12	ICE_C	?	Involved in mediating apoptosis following ER stress. Related to mouse caspase-1 and caspase-11 and human caspase-4 and caspase-5
Caspase-13	ERICE	?	Member of the ICE family of caspases that include caspase-1 and caspases-4, -5 and –11. Involved in inflammation.

Aside from the ligation of death receptors there are a number of other mechanisms through which the caspase cascade can be activated. Granzyme B can be delivered into cells by cytotoxic T lymphocytes and is able to directly activate caspases 3, 7, 8 and 10. The mitochondria are also key regulators of the caspase cascade and apoptosis. Release of cytochrome C from mitochondria can lead to the activation of caspase 9, and then of caspase 3. This effect is mediated through the formation of an apoptosome, a multi-protein complex consisting of cytochrome C, Apaf-1, pro-caspase 9 and ATP. The formation of the apoptosome is illustrated below.

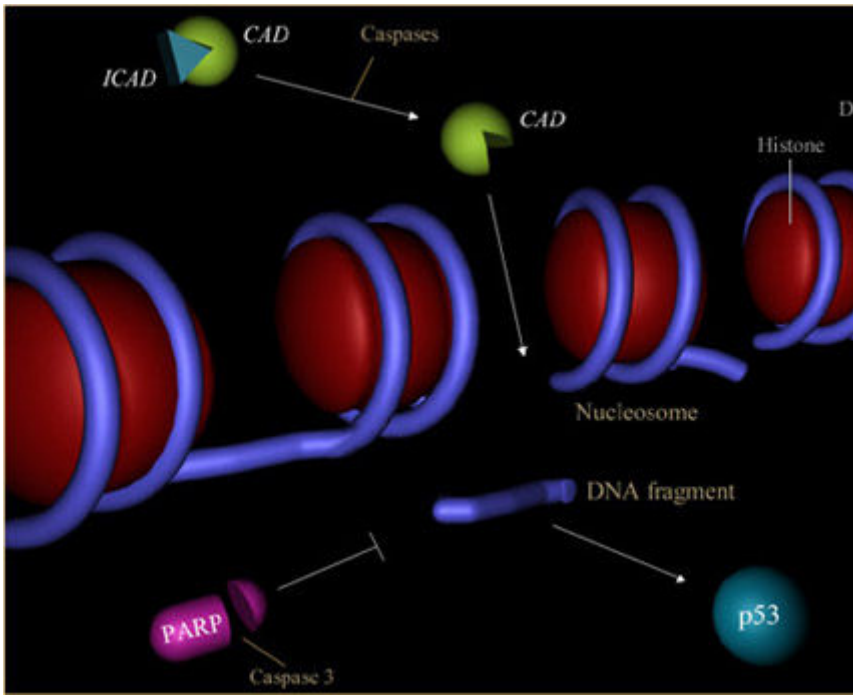


Caspase Activation

Cytochrome C released from the mitochondria binds to the cytosolic protein Apaf-1. This interaction results in a conformational change in Apaf-1 which, when stabilised by the binding of ATP, allows molecules of Apaf-1 to associate with each other. This results in the formation of a wheel-like structure that contains 7 molecules each of Apaf-1, cytochrome C and ATP. This wheel-like structure, known as the apoptosome, permits the recruitment of 7 molecules of procaspase-9 to the complex. The exact mechanism of caspase activation is still uncertain although two possibilities have been proposed. In one case the Apaf-1, cytochrome C and procaspase-9 complex can act as a stage to activate cytosolic procaspase-9 as it is recruited to the apoptosome. In the other scenario two apoptosome have been proposed to interact with each other and to activate the caspase-9 located on the other apoptosome.

Nuclear effects

One of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The degradation of DNA in the nuclei of apoptotic cells is accomplished in a number of ways following activation of caspases. The processes leading to DNA cleavage and nuclear changes are illustrated and described below.



Inactivation of enzymes involved in DNA repair.

The enzyme poly (ADP-ribose) polymerase, or PARP, was the first protein identified as a substrate for caspases. PARP is involved in repair of DNA damage and functions by catalyzing the synthesis of poly (ADP-ribose) and by binding to DNA strand breaks and modifying nuclear proteins. The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3.

Inactivation of enzymes involved in cell replication.

DNA topoisomerase II is a nuclear enzyme essential for DNA replication and repair. Caspases can inactivate this enzyme leading to DNA damage.

Breakdown of structural nuclear proteins.

Lamins are intra-nuclear proteins that maintain the shape of the nucleus and mediate interactions between chromatin and the nuclear membrane. Degradation of lamins by caspase 6 results in the chromatin condensation and nuclear fragmentation commonly observed in apoptotic cells.

Fragmentation of DNA.

The fragmentation of DNA into nucleosomal units - as seen in DNA laddering assays - is caused by an enzyme known as CAD, or caspase activated DNase. Normally CAD exists as an inactive complex with ICAD (inhibitor of CAD, also known as DNA fragmentation factor45). During apoptosis, ICAD is cleaved by caspases, including caspase 3,to release CAD. Since CAD is a DNase with a high specific activity (comparable to or higher than DNase I and DNase II) rapid fragmentation of the nuclear DNA follows.

Role of Nitric Oxide

Nitric oxide (NO) is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes including vasodilation , neuronal function, inflammation and immune function. Nitric oxide has also been demonstrated to be involved in the regulation of apoptosis. The effects of apoptosis vary depending upon the dose of NO and the type of cell used and has been shown to be able to both induce apoptosis and to protect from apoptosis in different cell types. Nitric oxide has been demonstrated to inhibit apoptosis in a number of cell types including leukocytes, hepatocytes, trophoblasts and endothelial cells. Generally the anti-apoptotic effects of NO can be mediated through a number of mechanisms such as the nitrosylation and inactivation of many of the caspases including caspase 3, caspase 1 and caspase 8. Other mechanisms include activating p53, upregulating heat shock protein 70 (and consequently blocking recruitment of pro-caspase 9 to the Apaf-1 apoptosome), upregulating Bcl-2 and Bcl-XL (with subsequent inhibition of cytochrome C release from the mitochondria) and activating cGMP signaling leading to activation of cGMP-dependent protein kinases and suppression of caspase activity. The effects of NO on apoptosis are generally classified as cGMP dependent or independent. Nitric oxide is able to activate cGMP signaling through the interaction of NO with the haem group of guanylate cyclase. The production of cGMP leads to the activation of cGMP-dependent protein kinases and possibly to increased expression of anti-apoptotic proteins. We are particularly interested in the anti-apoptotic effects of NO on endothelial cells and trophoblasts and are currently investigating the mechanism of action of NO in these cells.