MECHANISMS OF CELL DEATH

*"There is a time for everything, and a time for every work under heaven."*

*There is a time to be born, and a time to die;*

*the time when it is planted, and the time when it is uprooted;*

*A time to kill, and a time to heal;*

*the time when it breaks down, and the time when it is built up.*

*A time to cry and a time to laugh; a time to cry and a time to play;*

*The time when the stones are scattered, and the time when the stones are gathered;*

*the time to hug, and the time to leave the hug;*

*The time when it flows, and the time when it is lost; the time when it is kept, and the time when it is thrown away;*

*Time to tear, and time to sew; time to be silent and time to speak.*

*A time to love, and a time to hate; a time for war and a time for peace."*

**(Bible, Old Testament)**

In the event of a massive, irreversible error in the genome, anoxia or some signaling imbalance, a "death program" is initiated in the cell. This process takes place in the cell according to precisely defined rules. Cell death mechanisms are triggered to eliminate cells that have neoplastic potential, including cells that carry mutations that cause growth deregulation, and other cells that have suffered multiple genome damage. One of the characteristics of all types of malignant cells is the disruption of the function of the cell death program.

Cells die in (at least) three ways:

* suicide (apoptosis, cell death type I)
* autophagy (cell death type II)
* consequence of significant cell damage by physico-chemical agents (necrosis, cell death type III)

**Apoptosis**

**Apoptosis** is programmed cell death. It is the process of eliminating non-functional and unwanted cells. A cell can actively initiate its own death with the expenditure of energy. Apoptosis is an integral part of physiological processes, but also a response to certain pathological conditions. Apoptosis (dying from within, programmed cell death, cell suicide) is a highly regulated process that occurs in one cell independently of the surrounding cells. Light and electron microscopy detected many morphological changes of the cell during apoptosis. At the beginning of the process, cell shrinkage and pyknosis of the flagellum are visible with a light microscope. Cells lose volume, cytoplasm becomes denser and organelles are densely distributed. Pyknosis is the result of chromatin condensation, and the nucleus often takes the shape of a horseshoe (Figure 1). Cell shrinkage is a consequence of destruction of laminin and actin filaments of the cytoskeleton. Apoptotic cells become rounded due to these changes in the cytoskeleton. This is followed by the inversion of the cell membrane and the exposure of phosphatidylserine on the outside of the membrane. At the same time, the cell matrix is ​​destroyed, the nucleus is fragmented and the DNA is fragmented (Figure 2). This is followed by the swelling of the cell membrane and the separation of cell parts into apoptotic bodies ([Film 1: Apoptotic blebs](1%20blebs.wmv)). Blebs contain cytoplasm and densely packed organelles, as well as nuclear fragments. This is an energy dependent process. During apoptotic death, the cell expends energy to die. These changes take place at the cellular level and no intracellular contents are released. The exposure of phosphatidyl-serine on the outer surface of the cell membrane represents an early event in the process and serves as an "eat me" signal for surrounding phagocytes, which recognize it with receptors for damage patterns. Surrounding cells and tissue macrophages will recognize such cells, phagocytose them and safely remove them (preventing the development of inflammation). The inflammatory reaction is absent because the apoptotic cells do not release their contents into the intercellular space, and the apoptotic corpuscles are quickly phagocytosed.

Recent studies show that other proteins are also expressed on the surface of the cell membrane of apoptotic cells. Calreticulin is a protein that, together with phosphatidyl serine, is thought to facilitate recognition.



**Figure 1.** Morphology of the apoptotic cell

Cells that die by apoptosis undergo the following changes:

* shrinking
* cell membrane swelling
* nucleus condensation
* damage to mitochondria and release of cytochrome-c
* formation of apoptotic bodies
* detection of phosphatidyl-serine, normally hidden within the cytoplasmic membrane (eat me signal)
* phagocytosis by tissue macrophages
* phagocytes secrete anti-inflammatory cytokines (IL-10, TGF-β)

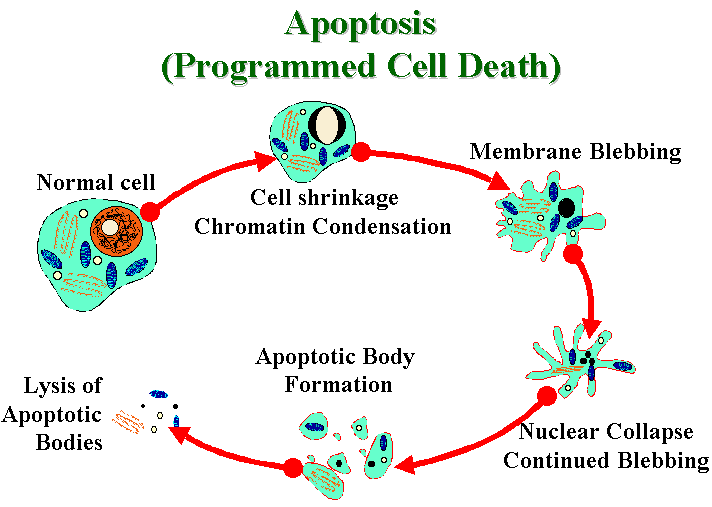
**The importance of apoptosis**

1. **organism development** (for the proper development of the organism, apoptosis is as important as mitosis):

* In the average human body, about 10 billion cells die by apoptosis every day. Apoptosis provides a balance between cell proliferation and death.
* The process of embryogenesis includes the formation of tissues and organs. In embryonic development, apoptosis supports the formation of organs and forms. Example: separation of fingers requires the removal of the tissue between (by the process of apoptosis).
* Both the nervous and immune systems arise through the excessive production of cells, which is followed by the death of those that failed to form a functional synapse or antigen receptor, all such "failed" cells die by apoptotic death.

1. The important role of apoptosis continues after birth:

* When migrating from the deeper parts of the skin to the surface, keratinocytes undergo apoptosis, turning into an outer protective layer of dead cells.
* Apoptosis is also accompanied by hormone-dependent involutional processes. Physiological decrement in the level of circulating hormones leads to the death of hormone-dependent cells (menstrual cycle, ovarian atrophy during menopause, prostate atrophy after castration)
* In the immune system, it enables the elimination of autoreactive T and B lymphocytes (tolerance). In the final phase of the immune response, effector cells are eliminated.
* Cells infected with viruses, cells with irreparable genetic mutations, cells damaged by radiation, cytostatics, antibiotics, oxidative stress, action of toxins, etc. they can initiate the process of self-destruction, or the process is initiated by cytotoxic T lymphocytes and NK cells.
* As the organism ages, some cells deteriorate faster and are removed by apoptosis. Oxidative stress is thought to play an important role in the pathophysiology of apoptosis, through mitochondrial DNA damage.



**Figure 2.** Morphological changes of cells during apoptosis

Apoptosis has been discovered many times and forgotten just as many times:

* In 1842, Vogt described cell death in amphibians for the first time.
* In 1885, almost half a century later, Fleming discovered the cell death of the rabbit's de Graaff follicle. This is the first discovered case of cell death as part of physiological functions. He explained cell death by lysis of chromosomes.
* The term apoptosis was used for the first time in 1972 in the now legendary paper of the Australian pathologist John Kerr. He rediscovered apoptosis as cell death of hepatocytes that is different from necrosis and called it shrinkage necrosis.

The first knowledge about the mechanisms involved in the process of apoptosis came from examining the development of the nematode *Caenorhabditis elegans* (nematodes are round worms). It was noted that the formation of an adult worm requires 1090 somatic cells, of which exactly 131 undergo apoptosis. These 131 cells die at precise moments during the development of the worm, neither the number of cells nor the time of death varies during the development of different individuals of the same species, which indicates the exceptional precision and control of this process.

Induction of apoptosis involves the activation of cytosolic enzymes called caspases. Caspases are cytoplasmic cysteinyl-aspartate-specific endoproteases that destroy essential structural components including the genetic material of the cell. They are so named because they cleave substrates with aspartic acid residues. Caspases are proenzymes (inactive form of enzymes) present in the cytoplasm of most cells. They are activated by a cascade of proteolytic cleavage, and the sequence of their activation depends on the way of initiation of apoptosis. This proteolytic cascade, in which one caspase activates the next, amplifies the apoptotic signal and consequently leads to rapid cell death. Today, 14 caspases are known, which are designated by ordinal numbers 1-14. Some caspases function as initiators, starting the process of apoptosis (2, 8, 9 and 10), some function as effectors or executioners, cleaving many substrates (3, 6 and 7), and some as inflammatory caspases (1, 4-7).

**Mechanisms of apoptosis**

Today, it is considered that there are two main pathways for inducing apoptosis:

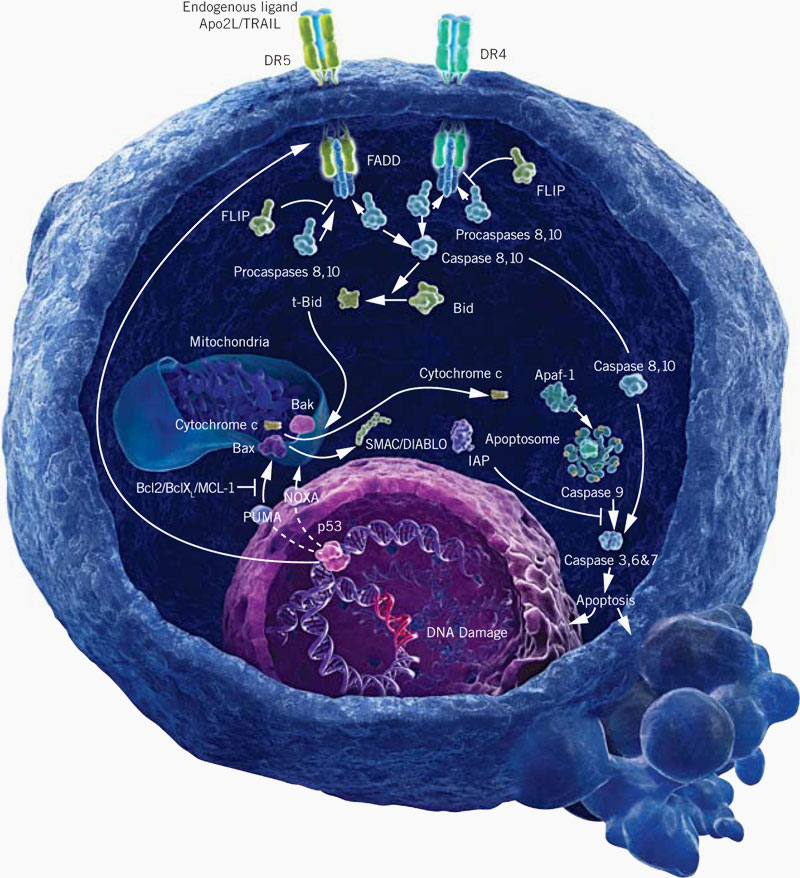
* External pathway (death receptors)
* Intrinsic pathway (mitochondria)

However, today it is also known that the two mentioned pathways are interconnected and that molecules engaged in one can trigger the other signaling pathway.

There is another pathway, which involves T-cell cytotoxicity, mediated by perforin and granzymes A or B.

***Cell death as a result of loss of survival stimuli: an internal signal***

Most cells need constant stimulation by other cells or constant adhesion to the surface on which they grow.The role of cytokines, growth factors and some hormones is particularly important.When the cell is deprived of the stimulus for survival, apoptosis occurs (Figure 3).



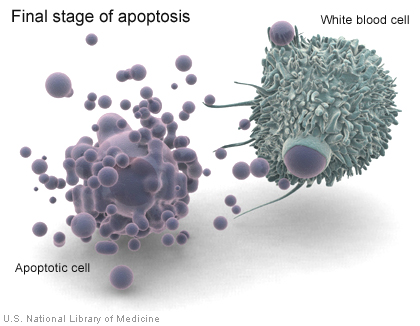
**Figure 3.** Mechanisms of apoptosis

DNA damage triggers apoptosis from the cell itself. This effect has: replication error, the effect of ionizing radiation, chemotherapeutics, drugs such as nucleoside analogues and numerous DNA-reactive toxins. Reactive oxygen radicals that arise as products of aerobic metabolism can activate apoptosis by affecting the permeability of the mitochondrial membrane.

All the mentioned stimuli cause a change in the integrity of the inner mitochondrial membrane, which results in the opening of the "mitochondrial permeability transition MPT pore", the loss of the mitochondrial transmembrane potential and the release of pro-apoptotic proteins into the cytosol. Those proapoptotic proteins are: Cytochrome-C and Smac/DIABLO and they activate the caspase-dependent pathway.

After release into the cytoplasm, cytochrome C forms a complex with an enzyme called apoptosis activating factor-1 (Apaf-1) with the expenditure of energy (ATP). The complex activates caspase-9, and together with it causes apoptosis. Apoptosis activates caspase 3, an effector protein that causes cell degradation by tearing the actin threads of the cytoskeleton, the cell separates, swells, protrusions on the cytoplasmic membrane) separate into bubbles, i.e. apoptotic bodies (blebs) (Figure 4).

Smac/DIABLO inactivate IAP (proteins that inhibit apoptosis).



**Figure 4.** Formation and elimination of apoptotic bodies

The control and regulation of the mitochondrial pathway of apoptosis activation is carried out by molecules that are members of the Bcl-2 family of proteins. These proteins control the permeability of the mitochondrial membrane. They can be pro-apoptotic and anti-apoptotic. To date, 25 genes in the Bcl-2 family have been identified. Some are responsible for the synthesis of anti-apoptotic (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG) and some for pro-apoptotic molecules (Bcl-10, Bax, Bak, Bid, Bad , Bim, Bik and Blk) ([Movie 2: Mitochondrial membrane permeability](2%20Mitochondria%20and%20apoptosis.wmv)).

Pro-apoptotic proteins bind to the mitochondrial outer membrane, altering its integrity. The result is an increase in the permeability of the mitochondrial membrane, which enables the entry of Ca2+ ions and the subsequent release of several types of proteins from the mitochondria, including cytochrome C.

The aforementioned pathways of apoptosis represent passive cell death. They do not require active signals from death receptors. They are also called death by neglect (referring to death that occurs when an external antiapoptotic signal is missing) because the cell will die by apoptosis if they are not protected by pro-survival stimuli.

***Cell death induced by death receptors: an extrinsic signal***

Another way of initiating apoptosis begins with the binding of signaling molecules-ligands to transmembrane receptors (Figure 5). The best described receptors (death receptors) belong to the tumor necrosis factor (TNF) family. The first identified among them was the receptor for TNF, and the family includes a large number of proteins, such as Fas, CD40 and others. These are homotrimeric molecules (a homotrimeric molecule implies that three identical units are connected in one complex entity, while, for example, a heterotrimeric molecule means that three different subunits are connected in such a complex) whose extracellular parts are rich in cysteine, and the cytoplasmic parts contain about 80 amino acids (these intracytoplasmic parts are also known as domains of death). These death domains play an important role in transmitting death signals generated at the cell membrane to the intracellular signaling system. Among the better-known ligands and corresponding death receptors, we distinguish FasL/ FasR, TNF-α/ TNFR1, Apo3L/DR3.

The series of events during extrinsic induction of apoptosis is best studied in the FasL/FasR and TNF-α/TNFR1 initiation models ([Movie 3: Ligands and death receptors](3%20Death%20Receptors.wmv)).

Binding of the death ligand (eg FasL) to the death receptor (Fas) enables receptor clustering - receptor trimerization. Trimerization takes place via the adapter protein FADD (Fas-Associated-Death-Domain-Protein). FADD binds and activates caspase-8. Active caspase-8 is able to activate effector caspases and thus trigger apoptosis. Namely, it activates caspase 3, which is an effector enzyme capable of initiating cell degradation. The apoptotic pathway via TNF ligand and receptor is similar to the one described. A special adapter protein TRADD binds to TNF receptor 1, which is capable of binding different proteins on the activated part of the receptor. If the activated part binds to FADD-death domains, procaspase 8 and the subsequent cascade of effector caspases will be activated.

Mitochondrial damage in the FasL/FasR signaling pathway is mediated by the activation of the pro-apoptotic Bid protein by caspase 8. This is one example of the intersection of the extrinsic and intrinsic pathways of apoptosis.

***Cell death induced by perforins and granzymes***

Cytotoxic T lymphocytes (CTL) and NK cells recognize and bind to cells infected with viruses. CTL and NK exert their cytotoxic effect on infected cells by secreting the enzyme perforin, which breaks down the cell membrane and creates pores through which other macromolecules secreted from the granules of these cells enter the infected cell. The most important among those secreted proteins are: serine proteases granzyme A and granzyme B.

Granzyme B can directly activate caspases 3, 7, 8 and 10 and thus trigger apoptosis. It has also been shown that granzyme B can trigger apoptosis via the mitochondrial pathway, by activating the Bid protein and subsequently releasing cytochrome C. Granzyme A, also important for T cell cytotoxicity, activates caspase-dependent apoptosis ([Movie 4: Apoptosis induced by cytotoxic T lymphocyte](4%20Apoptosis.wmv)).

**Autophagy**

Autophagy or autophagocytosis is an evolutionarily conserved catabolic process of degradation of cellular components (proteins and cellular organelles) by lysosomal machinery. The process begins with the formation of autophagosomes (vacuoles with a double membrane) in the cytosol, which surround and draw in cell organelles and cytoplasm. They then fuse with lysosomes to form autolysosomes where the engulfed cellular components are degraded and recycled for protein synthesis and ATR (Figure 5). There are several subtypes of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy. Microautophagy is a form of autophagy in which cytosolic structures are directly taken over through invaginations on the lysosomal membrane.

Macroautophagy involves first the formation of a vacuole and then its subsequent fusion with the lysosome in a structure called autolysosis.

Shaperone-mediatedautophagy requires the presence of Hsc-70 protein.

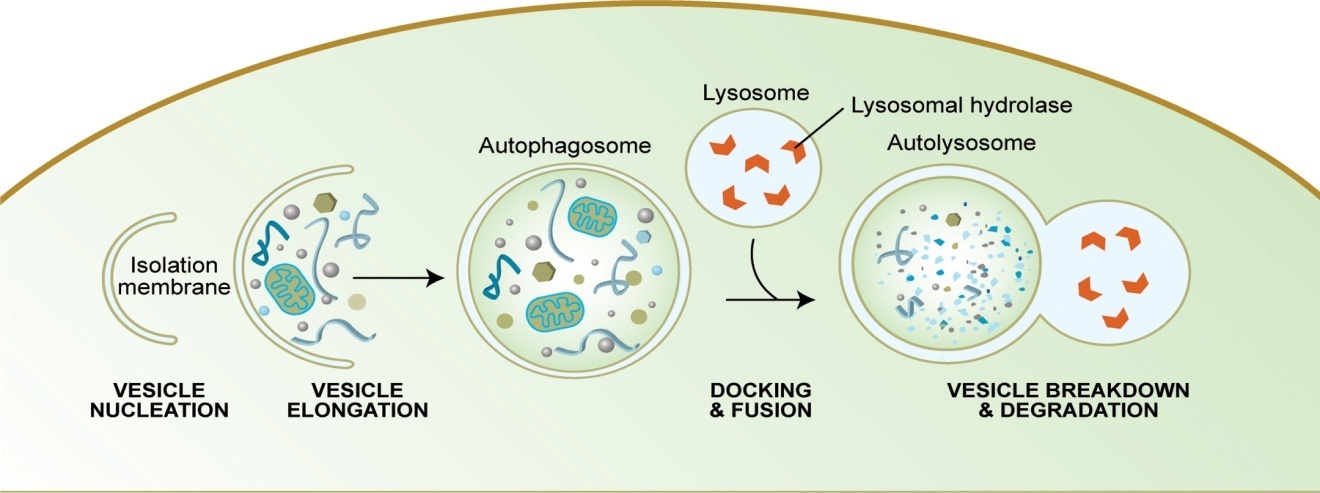


Figure 5. Autophagy process

The process of autophagy is regulated through the PI3K/AKT/mTOR signaling pathway (eng. PI3 kinase, Phosphatidyl Inositol 3 kinase; AKT- serine/threonine kinase which, when activated by PI3-kinase, activates mTOR- mammalian target of Rapamycin), which represents the link of availability nutrients to cells and cellular metabolism. In the absence of nutrients in normal cells, the RI3-kinase/AKT signaling pathway is activated, which stops protein synthesis and at the same time activates the catabolic processes of autophagy. Autophagy can also be triggered as a result of the accumulation of reactive forms of oxygen, due to hypoxia and bacterial infection, and enables tumor cells to survive if they are deprived of nutrients.

**Importance**

Autophagy enables the short-term survival of cells deprived of nutrients by recycling their own nutrients/building blocks.

Autophagy can induce cell death by progressive consumption of cellular components - programmed cell death type II.

Both normal and tumor cells "use" autophagy to survive metabolic stress (lack of nutrients and building materials). Autophagy is a mechanism that protects cells from infections by intracellular microorganisms (innate immunity) but also intensifies the survival and proliferation of T lymphocytes (acquired immunity). Autophagy plays an important role in cell differentiation. Dysfunction in autophagy can be associated with neurodegenerative diseases, inflammatory bowel diseases and cancer.

Autophagic cell death, Cell death type II, cytoplasmic cell death.

Autophagy is also a form of cell death, when most cellular components in the cell are broken down and programmed cell death is induced in cells that cannot "enter apoptosis". It is not always clear whether autophagy is involved in the initiation or effector stages of cell death or simply represents a failed survival case of a cell deprived of nutrients/building blocks.

**Necrosis**

It represents the III type of cell death. Necrosis is an accidental, uncontrolled process. Cell necrosis occurs as a result of significant cell damage caused by various physical and chemical agents such as: hypoxia, extreme temperatures, complement action... All these agents damage the cell membrane until it ruptures, which results in cell swelling (osmotic pressure). If the damage is large, such a cell cannot maintain fluid and electrolyte balance (Na and K ions enter the cell uncontrollably). This is followed by random destruction of cellular structures (Figure 6). The consequent loss of cell membrane integrity results in the release of cytoplasmic contents into the surrounding tissue. The released cellular proteins (enzymes) reaching the intercellular space are a kind of chemoattractants that trigger an inflammatory reaction followed by the entry of leukocytes into the tissue. Inflammation helps limit possible infection, but also damages tissues. Therefore, necrosis usually involves a large number of cells.

**During necrosis the cell spends energy to survive (unlike apoptosis) trying to remove the damage and restore osmotic balance.**

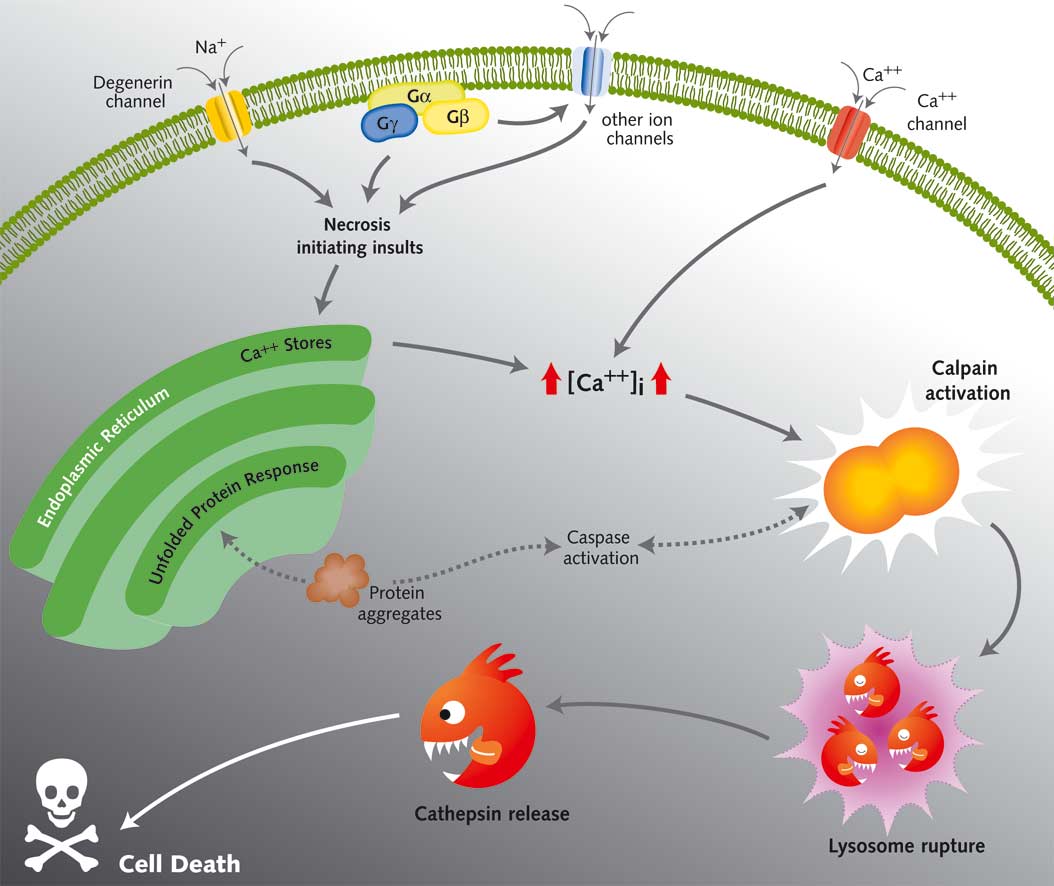


Figure 6. Mechanism of necrosis

Cells damaged by physical and chemical agents go through a series of characteristic changes:

* cells (and organelles) swell, because the ability of the cell membrane to control fluid and electrolyte balance is impaired
* cell contents are released and reach the intercellular space, which is the cause
* inflammation of the surrounding tissue

Necrosis is an uncontrolled and passive process that usually affects a large number of cells, while apoptosis is a controlled and energy-dependent process that affects one or several cells.

Cell death with or without an accompanying inflammatory response is one of the best ways to distinguish between apoptosis and necrosis (Figure 7).

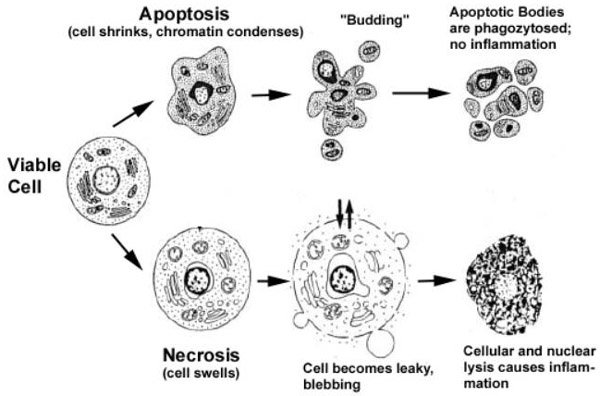


Figure 7. Morphological differences between necrosis and apoptosis

**Necroptosis**

Necroptosis is a form of programmed necrosis. It plays an important role in embryonic growth and development, represents a defensive mechanism against intracellular pathogens (viruses), and at the same time plays an important role in the pathophysiology of myocardial infarction, stroke, atherosclerosis, ischemia-reperfusion injury, pancreatitis and inflammatory bowel disease. Necroptosis is also a mechanism to prevent infection with viruses that inhibit apoptosis.

It is characterized by a change in the permeability of the plasma membrane and the release of cell contents and molecules that are labeled as damage-associated molecular patterns (DAMPs). DAMPs are a family of molecules that, released from the cell, can trigger an immune response.

The list of physiological and pathological stimuli that trigger necroptosis is constantly increasing, and these are various ligands that bind to receptors designated as death receptors, then the so-called microbe-associated molecular patterns, MAMPs, which represent components of microorganisms that usually alert the immune system to the development of an infection.

Central molecules in the process of necroptosis are RIP kinases (eng. Receptor-interacting protein kinase, RIP), which are serine-threonine kinases. In their active form, RIP kinases build the Necrosome, a structure that plays a key role in the initiation of the necroptosis process. However, under normal conditions, when caspases are active, necrosis does not form because caspase 8 cleaves active RIP kinases. Death receptors, including TNFR1 and Fas cause activation of RIP kinases. Different cellular stimuli: TNF, Fas ligand, double-stranded RNA (dsRNA), interferon-γ and ischemia-reperfusion injury, induce necrosis that follows defined steps and signaling events reminiscent of apoptosis. This regulated necrosis is called necroptosis.