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ON

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Course Manual

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PROGRAMMED CELL DEATH—APOPTOSIS IN HEALTH AND DISEASE

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Introduction

The health of multicellular organism depends not only on the body's ability to produce new cells but also on the ability of individual cells to destruct when they become superfluous or disordered. Millions of cells are sacrificed every hour. This critical process is essential for the development and maintenance of multicellular organism; is termed apoptosis. The significance of apoptosis was overlooked for decades. But biologists have recently made rapid strides in understanding how cellular suicide is enacted and controlled. It is known that aberrant regulation of apoptosis-leading to too much or too little apoptosis-probably contributes to disorders like cancer, AIDS, Alzheimer's disease and autoimmune diseases (rheumatoid arthritis, lupus erythromatosis).

Death is not bad for the body always. In fact it is necessary for the health and well being of multicellular organism. The tadpole deletes its tail during transformation to frog; the arthropods delete several appendages/tissues during metamorphosis; mammals erase countless neurons as the nervous system takes shape. Human embryo deletes the web between digits during development. The lens of eye, which forms during embryonic development, consists of apoptotic cells that have replaced their internal contents with protein crystalline. Cells composing intestinal villi arise at the base of the villi and over several days, travel to the tip. They die there and are sloughed off. Skin cells begin life in the deepest layers and then migrate to the surface, undergoing apoptosis along the way. The dead cells forms skin's protective layer. The cells lining uterine wall perish by apoptosis during menstruation. The cells that become infected by virus or sustain irreparable genetic mutation often kill themselves. T lymphocytes during the developmental stage encountering an antigen die by apoptosis.

Necrotic death occurs when a cell is severely injured, by physical injury or by oxygen/ nutrient deprivation. This death has the following features. The cell and its internal organelles undergo severe swelling/ ballooning due to failure of ion pumps. Another hallmark of necrosis is inflammation bringing white blood cells to the site of necrosis. Inflammation also damages the normal tissue in the vicinity. 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). Cells that are induced to commit suicide: shrink, have their mitochondria break down with the release of cytochrome c, develop bubble-like blebs on their surface and have the chromatin (DNA and protein) in their nucleus degraded. The chromatin breaks into small, membrane-wrapped, fragments. The phospholipid phosphatidylserine, which is normally hidden within the plasma membrane, is exposed on the surface. This is bound by receptors on phagocytic cells like macrophagesand dedritic cells, which then engulf the cell fragments.

The [illegible] specific signals [illegible] 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.

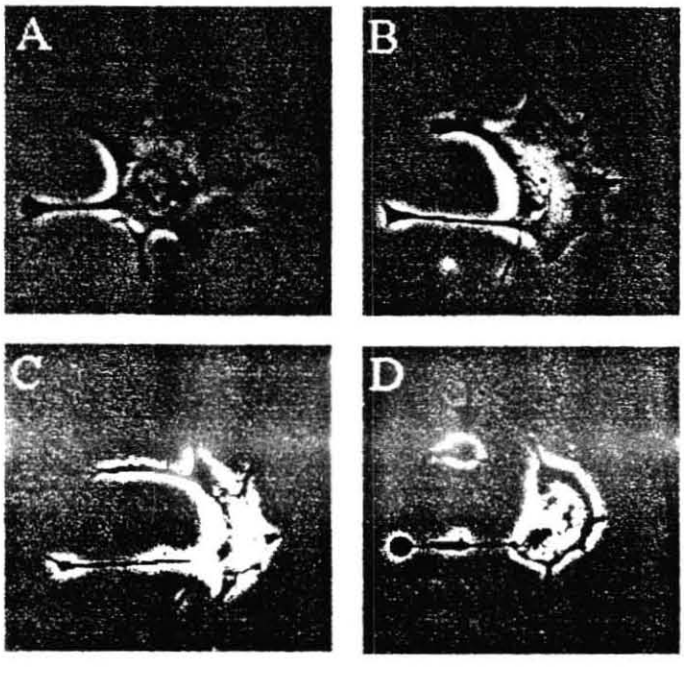

Fig1

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). In apoptosis cell spends energy, as it is an active process initiated by cell's own cleaving enzymes, which are interleukin-1 converting enzyme like proteases (ICE like proteases).

The Mechanisms of Apoptosis

There are three pathways through which apoptosis mechanism is activated. One generated by signals arising within the cell. Another triggered by **death activators** binding to receptors at the cell surface. These are TNF-□ Lymphotoxin and Fas ligand (FasL). A third that may be triggered by dangerous reactive oxygen species

Apoptosis triggered by internal signals: the intrinsic or mitochondrial pathway

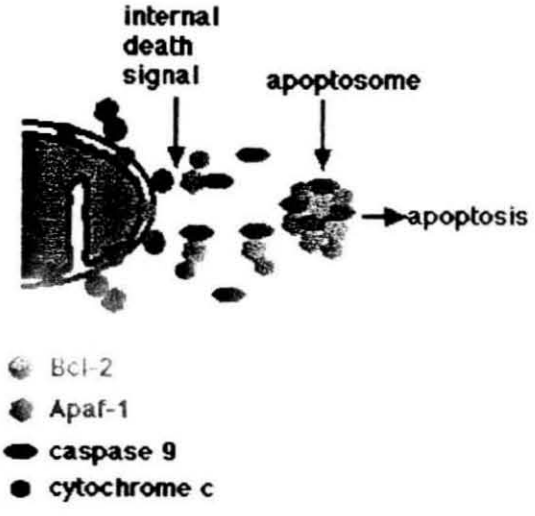

Fig.2

In a healthy cell, the outer membranes of its mitochondria express the protein **Bcl-2** on their surface. Bcl-2 is bound to a molecule of the protein **Apaf-1**. Internal **damage** to the cell (e.g., from reactive oxygen species) causes Bcl-2 to release Apaf-1 to no longer keep cytochrome c from leaking out of the mitochondria. The released cytochrome c and

Apaf-1 bind to molecules of **caspase 9**. The resulting complex of **cytochrome c, Apaf-1, caspase 9** (and ATP) is called the **apoptosome**. These aggregate in the cytosol. Caspase 9 is one of a family of over a dozen caspases. They are all proteases. They get their name because they cleave proteins - mostly each other - at aspartic acid (Asp) residues). Caspase 9 cleaves and, in so doing, activates other caspases. The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity (rather like that in blood clotting and complement activation), which leads to digestion of structural proteins in the cytoplasm degradation of chromosomal DNA and phagocytosis of the cell.

Apoptosis triggered by external signals

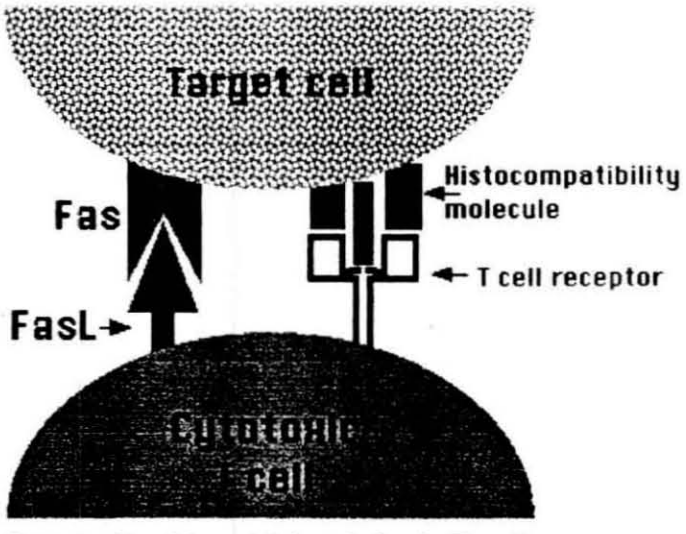

Fig.3

One method by which cytotoxic T cells induce their targets (e.g., virus-infected cells) to commit suicide (apoptosis)

Fas and the **TNF receptor** are integral membrane proteins with their receptor domains exposed at the surface of the cell. Binding of the complementary **death activator** (**FasL** and **TNF** respectively) transmits a signal to the cytoplasm that leads to activation of **caspase 8**. Caspase 8 (like caspase 9) initiates a cascade of caspase activation leading to Phagocytosis of the cell. When cytotoxic T cells recognize (bind to) their target, they produce more **FasL** at their surface. This binds with the **Fas** on the surface of the target cell leading to its death by apoptosis.

Apoptosis-Inducing Factor (AIF)

Neurons, and perhaps other cells, have another way to self-destruct that - unlike the two paths described above - does not use caspases. Apoptosis-inducing factor (**AIF**) is a protein that is normally located in the inter membrane space of mitochondria. When the cell receives a signal telling it that it is time to die, AIF is released from the mitochondria (like the release of cytochrome c in the first pathway) migrates into the nucleus and binds to DNA, which triggers the destruction of the DNA and cell death.

There are a number of mechanisms through which apoptosis can be induced in cells. The sensitivity of cells to any of these 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. When cells are subjected to radiation injury or agents that induce mutation, the damage spurs the cells to a protein called p53. This protein activates the suicide pathway.

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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.

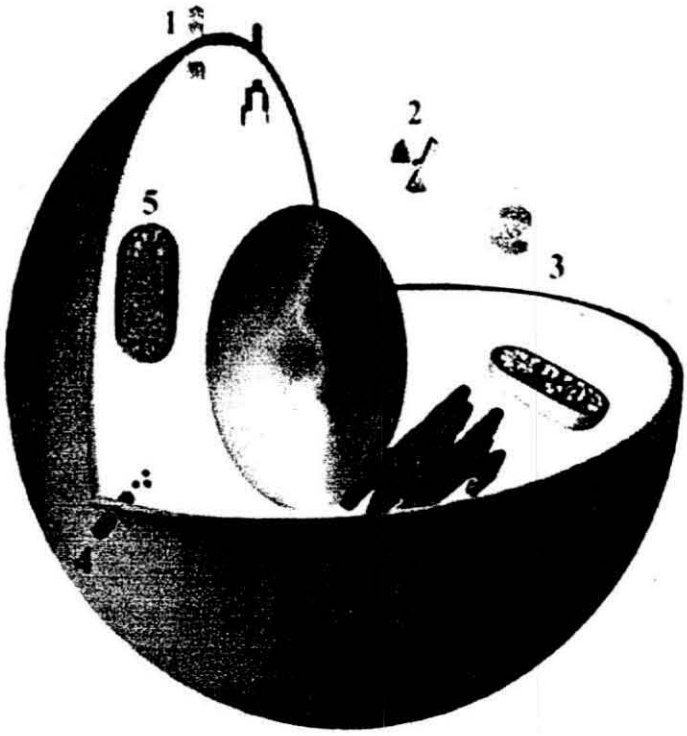

Fig.4

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), TNFR 1(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 (seeFigure). 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.

Viral diseases and cancer

Two human papilloma viruses (**HPV**) have been implicated in causing cervical cancer. One of them produces a protein (E6) that binds and inactivates the apoptosis promoter **p53**. **Epstein-Barr Virus** (**EBV**), the cause of mononucleosis and a cause of Burkitt's lymphoma It produces a protein similar to Bcl-2 and produces another protein that causes the cell to increase its own production of Bcl-2. Both these actions make the cell more resistant to apoptosis (thus enabling the cancer cell to continue to proliferate). Even cancer cells produced without the participation of viruses may have tricks to avoid apoptosis.

Some **B-cell leukemias** and lymphomas express high levels of **Bcl-2**, thus blocking apoptotic signals they may receive. The high levels result from a translocation of the *BCL-2* gene into an enhancer region for antibody production **Melanoma** (the most dangerous type of skin cancer) cells avoid apoptosis by inhibiting the expression of the gene encoding **Apaf-1**.

Some cancer cells, especially lung and colon cancer cells, secrete elevated levels of a soluble "decoy" molecule that binds to FasL, plugging it up so it cannot bind Fas. Thus, cytotoxic T cells (CTL) cannot kill the cancer cells.

Other cancer cells express high levels of **FasL**, and can kill any cytotoxic T cells (CTL) that try to kill them because CTL also express Fas (but are protected from their own FasL).

Apoptosis and Organ Transplants

For many years it has been known that certain parts of the body like, the anterior chamber of the eye and the testes are "immunologically privileged sites". Antigens within these sites fail to elicit an immune response. It turns out that cells in these sites differ from the other cells of the body in that they express high levels of **FasL** at all times. Thus antigen-reactive T cells, which express **Fas**, would be killed when they enter these sites.

This finding raises the possibility of a new way of preventing graft rejection. If at least some of the cells in a transplanted kidney, liver, heart, etc. could be made to express high levels of FasL, they might protect the graft from attack by the T cells of the host's cell-mediated immune system. If so, then the present need for treatment with immunosuppressive drugs for the rest of the transplant recipient's life would be reduced or eliminated. So far, the results in animal experiments have been mixed. Allografts engineered to express FasL have shown increased survival for kidneys but not for hearts or islets of Langerhans.

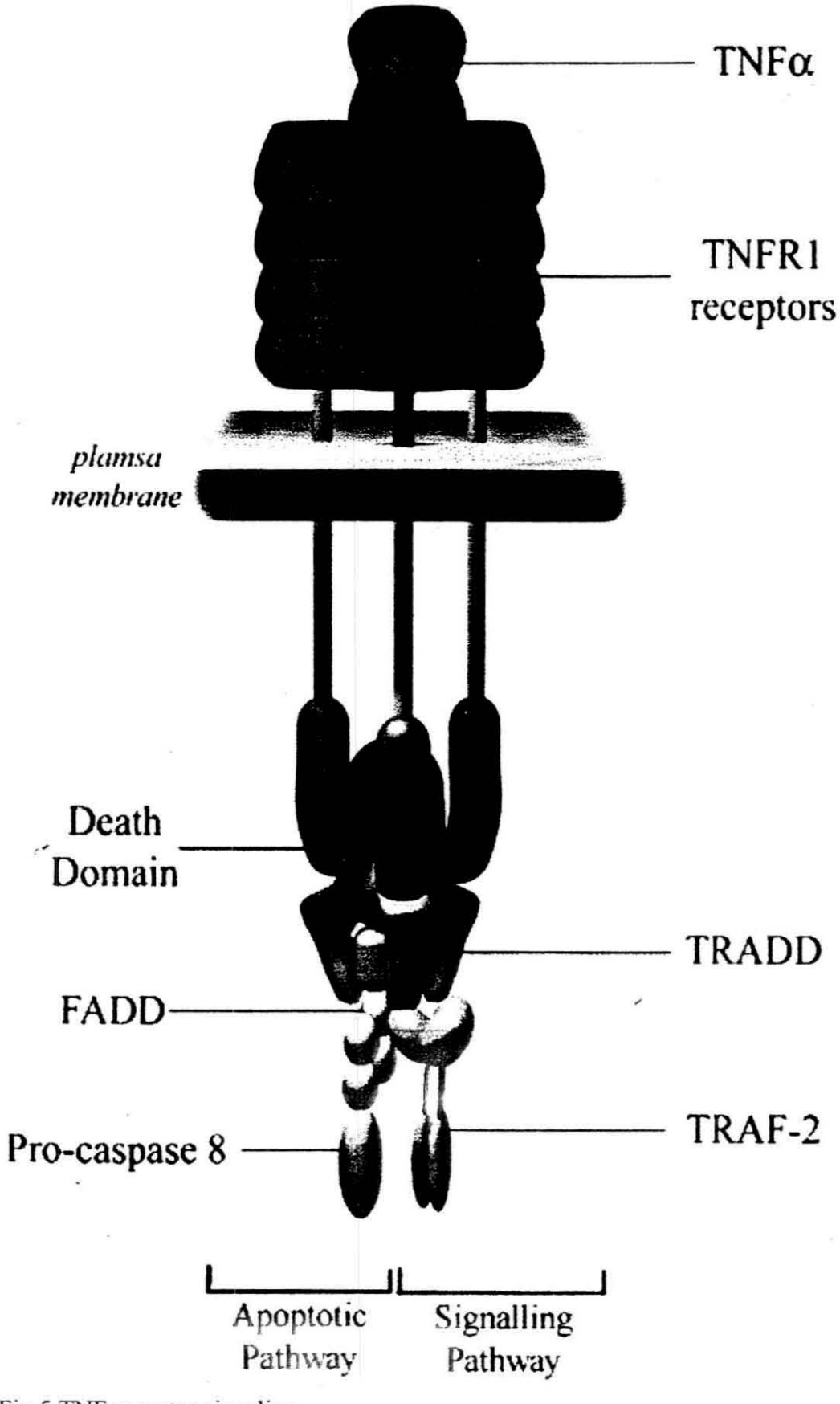

Fig.5 TNF receptor signaling

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. 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 associate 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.

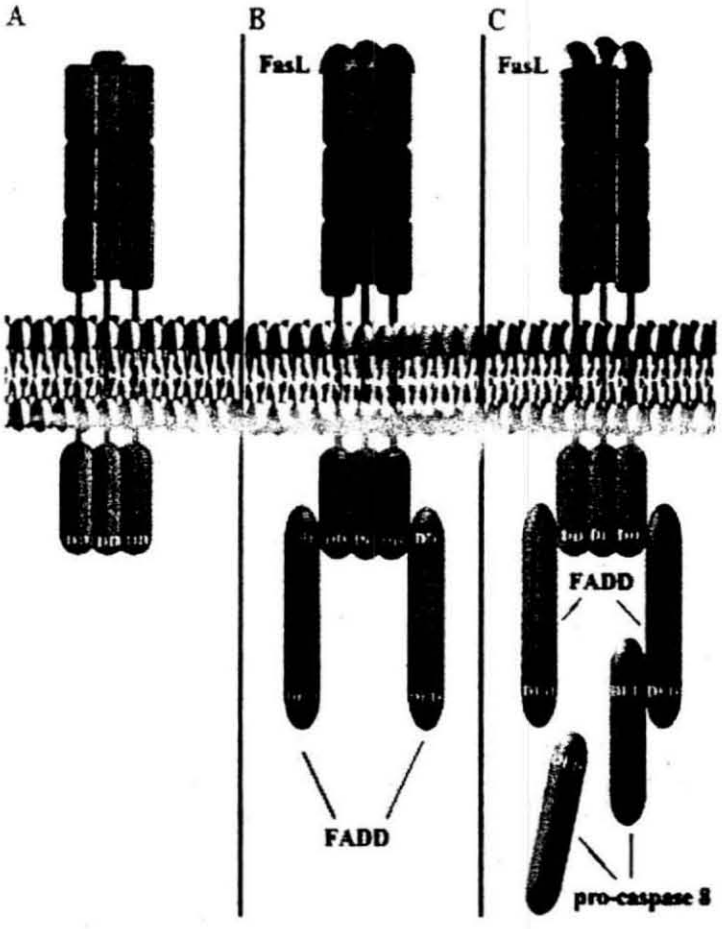

Fig.6 Activation of apoptosis through CD95 / Fas

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.

Bcl-2 proteins

The bcl-2 proteins are a family of proteins involved in the response to apoptosis. Some of these proteins (such as bcl-2 and bcl-XL) are anti-apoptotic, while others (such as Bad or Bax) are pro-apoptotic. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic bcl-2 proteins. When there is an excess of pro-apoptotic proteins the cells are more sensitive to apoptosis, when there is an excess of anti-apoptotic proteins the cells will tend to be less sensitive.

The pro-apoptotic bcl-2 proteins are often found in the cytosol where the act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome C and other pro-apoptotic molecules from the intermembrane space. This in turn leads to the formation of the apoptosome and the activation of the caspase cascade.