CHAPTER II

THEORY AND LITERATURE REVIEW

Apoptosis

Apoptosis is one of many cellular controlling processes, viz. cell proliferation and differentiation. It is particularly important in the development process, the effective functioning of the immune system,[20] and in some pathological conditions such as cancer, SLE, degenerative diseases, and AIDS.

Necrosis and apoptosis are the modes of cell death processes in living organisms.[21,22] Necrosis is a pathological cell death and occurs during the extreme cases of cell injury and repair.[22] Apoptosis is a normal physiological cell death process which occurs in all living organisms in their embryonic or adult development, thus eliminating unwanted functionally abnormal or harmful cells.[23] Apoptosis does not result from injury ; it is not reversible or harmful to organism but may be necessary for its normalcy. Defective regulation of apoptosis may result in the etiology of various diseases.[24]

In 1972 , Kerr and his coworkers described the ultrastructure of cell death called as shrinkage necrosis.[25] It was first coined by them as "Apoptosis"[26] derived from ancient Greek, "Hippocratic corpus" for falling off of tree leaves. Normal cell, programmed cell death (PCD), physiological cell death and cell suicide are the other terms used synonymously with apoptosis.

A cell will undergo apoptosis as a result of information integration after receiving signals from its environment and interpreting with internal information, e.g., its cell type, state of maturity, and developmental history. The external triggers include disappearance of trophic cytokines or hormones. The intracellular signals involved in induction of apoptosis are often associated with promotion of proliferation or differentiation in other cellular contexts, although certain intracellular mediators, e.g., Fas mediated pathway, are of particular importance in controlling apoptosis in certain cell types.[27]

The most important differences between necrosis and apoptosis are shown in Table 1.

Table 1. General differences between apoptosis and necrosis[28]

Characteristics	Apoptosis	Necrosis
Stimuli	Physiological	Pathological (injury)
Occurrence	Single cell	Groups of cells
Reversibility	No (after morphological change)	Yes (up to the point of no return)
Cytoplasmic organelles	Late stage swelling	Very early stage swelling
Lysosomal enzyme release	Absent	Present
Nucleus	Convolution of nuclear outline and breakdown	Disappearance

Characteristics	Apoptosis	Necrosis
DNA breakdown	Internucleosomal	Randomized
Cell	Formation of apoptotic bodies	Swelling and later disintegration
Phagocytosis by other cells	Present	Absent
Exudative inflammation	Absent	Present
Adhesions between cells and to basement membrane	Lost (early)	Lost (late)
Scar formation	Absent	Present

1. Occurrence

The proper development of multicellular organism depends on the elimination of selected cells through apoptosis. The roundworm (*Caenorhabditis elegans*), which is just a millimeter long, eliminates precisely 131 cells of its initial 1090 cells, as its hermaphrodite form matures to an adult. As tadpole becomes a frog, it deletes its tail cells. Human embryo are thought to use apoptosis to remove webbing between digits.[30]

Apoptosis provides an efficient mechanism of eliminating unwanted cells during normal embryonic and adult development.[31] These are usually normal cells that become unwanted for any one of a number of reasons.[32] Apoptosis has also been shown to occur in adult somatic cells during the course of normal tissue turnover,[23] the formation of keratinocytes, shedding of the intestinal lining, atrophy of the prostate after castration.[26] These include the epithelium of adrenal cortex,[33] endometrium,[34] the target cells of cytotoxic T cells, lactating mammary gland regression,[20] and ovarian follicle atresia.[35]

Apoptosis has extensively been studied in the immune system where it appears to play an important role in the deletion of self-reacting lymphocytes.[36] Cytotoxic T-lymphocyte,[37] K cells and NK cells[38,39] have been described to induce apoptosis to their targets.

2. Characteristics of apoptosis

2.1 Morphological aspects

The morphological features of necrosis[22,26] include dilatation of endoplasmic reticulum, increase in mitochondrial volume, flocculation of nuclear chromatin and cell swelling resulting in osmotic rupture of the cell. Thus, necrosis is a passive process that does not require any active participation of the cell in its own death.[40]

In contrast to necrosis, there is no inflammatory reaction and organelle swelling in apoptosis.[41] The cell passes through a series of morphologically distinct stages in their pathway to death. In the initial phase, an individual cell chromatin gets condensed resulting in the

fragmentation of cellular DNA. The cell shrinks due to loss of cytoplasmic volume and condensation of cytoplasmic proteins. Most of the intracellular organelles remain intact.[42,43]

The second phase is characterized by membrane blebbing leading to cellular fragmentation and formation of apoptotic bodies frequently containing nuclear remnants.[41,43,44] In the final phase, the neighboring cells and macrophages phagocytose the fragments for complete degradation. The apoptotic leakage of intracellular macromolecules does not elicit any inflammatory response and, hence, there is no secondary damage to the adjacent cells and no residual scar formation.[45]

2.2 Cytoplasmic changes

One of the most noticeable morphological features of apoptosis is the fragmentation of cell into apoptotic bodies. A rearrangement of the microfilament network of the cell must occur during this process as evidenced by the role of microtubule-disrupting agents such as colchicine, vinblastine, and nocodazole. All induce apoptosis suggesting that disruption of the microtubule network initiates events which lead to apoptosis.[46]

2.3 Chromatin changes

In 1980, a land mark paper by Wyllie was published revealing that glucocorticoids induce extensive DNA degradation in rat thymocytes *in vitro* during the onset of cell death.[47] The DNA degradation occurred in a very specific pattern producing fragments of DNA that were multiples of 180-200 base pairs. This is the length of DNA wrapped around the histone octamer in a nucleosome, which indicates that the chromatin is being cleaved at the linker DNA between nueleosomes, producing oligonucleosomal fragments. It occurs in almost all instances of apoptosis that has been studied. These include uterine epithelium,[34] prostate,[48] erythroid progenitor cells,[49] keratinocytes, peripheral blood lymphocytes.[50]

Ligation of some members of Fas/TNFR family promotes apoptosis but other promote cell survival.[51] It is a mechanism relevant to the regulation of immune system,[51] especialy T cell receptor.[52]

2.4 Cell membrane alteration in apoptosis

During apoptosis, there are extensive cell membrane alterations. The cell detaches from neighboring cells, from the culture substrate, *in vitro,* or from the extracellular matrix, *in vivo,* and the membrane loses specialized structure villi. Phagocytic cells may also recognize apoptotic cells by a change in the lipid composition of their outer plasma membrane. Normally anionic phosphatidylserine (PS) is located in the inner plasma membrane, while the neutral phospholipids

sphingomyelin and phophatidylcholine are found on the outer membrane bilayer. During apoptosis, PS is exposed and found on the outer membrane bilayer in order to elicit a phagocytic response.[53,54]

2.5 Biochemical aspects

The key molecules in the induction of both apoptosis and proliferation are protein kinase C, ceremide, c-myc, and p53, which reflect in a part of the adaptation of many signaling pathways in controlling different responses in different cell types and under different conditions. The cell-surface receptor mediated mechanisms which control apoptosis often act through a signal transduction system, i.e, through the stimulation of receptor, activation of protein kinase/phosphatase cascade and release of second messengers to upregulate or suppress the transcription of specific genes. The signaling pathways can intersect or crosstalk and, therefore, greatly alter a cell's response to a given stimulus.[45]

The nearly universal presence of internucleosomal cleavage in apoptotic cells suggests that there may be a common mechanism by which apoptotic occurs in different cell types and also the DNA fragmentation is an important part of the cell death mechanism. To support the fact that chromatin cleavage is a key step in the apoptotic process, a treatment of thymocytes with the nuclease inhibitor aurintricarboxylic acid and Zn^{2+} blocks DNA degradation and cell death induced by glucocorticoids and other agents. By the time, the DNA cleavage occurs, the cell is irreversibly committed to apoptosis and when

DNA is blocked the cell does not die. The relationship strongly suggests that DNA fragmentation is the cause of death in these cells.[40,45]

3. Molecular aspects of apoptosis

3.1 Death genes

As mentioned, the genetic analysis has identified two *C. elegans genes*, CED-3 and CED-4 that must function in the dying cells. Both genes act autonomously to cause PCD. Cell death was also blocked by using the antisense approach.[55] CED-3 encodes a many potential phosphorylation sites, wheareas CED-4 encodes a novel protein with two potential calcium binding domains, but it remains unknown how either protein participates in cell death.[40,56]

Similarily, a number of genes have been shown to be essential for apoptotic cell death in mammalians. The protooncogene, *c-myc*, that normally stimulates cell division is also involved in induction of apoptosis.[57] The increased expression of *c-myc* RNA and protein occurs early in apoptosis of fibroblasts.[58]

The tumor suppresser gene, *p53*, is another important gene belonging to this class, and the increassed levels of p53 protein is associated with apoptotic cell death.[59] The germline disruption of p53 gene results in the total disruption of apoptosis in the mammalian cells.[60,61]

3.2 Survival genes

There is another class of genes that normally act as a brake on the death program. If these genes are inactivated by mutation, many cells that would normally live undergo apoptosis. *C. elegans* CED-9 is such a gene of interest as it seems to act as an antagonist on the suicide program.[32] When its function is abnormally activated by mutation, the cell death does not occur. On the other hand, upon inactivation of CED – 9 gene by mutation, many cells that normally survive start undergoing CED – 3 and CED – 4 dependent apoptosis and the animal dies early during development.[60]

The existence of such antiapoptotic genes (survival genes) in mammalian cells has also been demonstrated.[61] The bcl-2, an inner mitochondrial membrane protein[61] not only inhibits apoptosis when overexpressed in a variety of mammalian cell, but the human gene can also suppress PCD in *C. elegans* when it is introduced into the worm.[62] However, unlike other oncogenes, bcl-2 does not stimulate cell proliferation but promotes survival of cells in a non-cycling state. Bcl - 2 inhibits induction of apoptotic cell death by *c-myc* and also renders cells less sensitive of radiation and cytotoxic drugs.[63]

Hence, the activity of a number of genes appears to be essential for the apoptotic cell death and it has been conserved in evolution from worms to humans, confirming that PCD is a fundamental feature of animal cells. However, this does not designate them as killer genes because of a gene to be considered as a killer gene, its product should alone be sufficient to induce the death of an other wise viable cell and should normally be

expressed during PCD. The killer genes may be expressed ectopically in naive cells and normally utilized during other physiological processes. Lethality may then result either from theirs overexpression or co-expression with other genes.[40]

Genetic studies in the nematode *C. elegans* showed that there are three important genes (CED-3, CED-4, CED-9) involved in controlling apoptosis during the development of worm. CED-3 and CED-4 promote apoptosis whereas CED-9 inhibit apoptosis. CED-4 binds to CED-3 and promotes the activation of CED –3, whereas CED-9 binds to CED-4 and prevents it from activating CED-3. Normally, CED-9 is complexed with CED-4 and CED-3, keeping CED-3 inactive.[64-66]

Human homologues of CED-3 gene encode members of the caspase family of proteases. Caspase, a familly of cysteine proteases with aspartate substrate specificity, is produced in cell as catalytically inactive zymogens which becomes activated by another caspase or by an autocatalytic mechanism, and the active caspase can in turn activate other caspases resulting in a cascade process.[67,68] CED-9 is homologue of the mammalian Bcl-2 family of antiapoptosis. Bcl-2 family includes two subgroups of proteins that either inhibit (Bcl-2, Bcl-x_L, Bcl-w) or promote (Bax, Bak, Bid, Bad) apoptosis.[69,70] The human homologue of CED – 4 is Apaf-1 (Apoptosis activating factor 1) which is required for the activation of caspase-3. Two Apafs have been identified : Apaf-2 is cytochrom c, and Apaf-3 is caspase-9.[71]

Apoptotic death can be triggered by a variety of stimuli, and not all cells necessarily will die in response to the same stimulus. Any agent or set

of condition that stress the metabolism or normal response mechanisms of a cell is likely to trigger the process of apoptosis. However, the level of stress is crucial. At the high stress levels, cells will die by necrosis, because they have no time to respond to the stimulus and die instantly. Examples include high levels of toxins, a sharp change in pH and high agitation rates. At the intermediate levels of cell stress, the cell is injured but not killed. The cell has time to activate its own death program. Thus, the cell dies in a controlled way, by apoptosis. At the low levels of environmental stress, cell can switch on the production of heat shock proteins which enable them to survive until the stress is removed. However, once a certain stress threshold is passed and survival is deemed impossible. Cell will die by apoptosis.[72]

Triggers of apoptosis include deprivation of growth factors, presence of receptor-ligand complexes on the cell surface, toxins, hyperthermia, viruses, free radicals, irradiation, and chemotherapeutic drugs. These signals can be divided as being external and internal signal.[71]

4. The external signal

The external signal is the presence of a family of receptors belonging to the tumor necrosis factor (TNF) receptor superfamily such as the Fas antigen, also known as APO-1/CD.[73] Fas is a cell surface protein that has been shown to initiate a signal for apoptosis when cross linked with Ligand (FasL) or specific antibody.[74] FasL is a homotrimeric molecule and each FasL trimer binds three Fas molecules.[75] Because some death domains have a propensity to associate with one another, Fas ligation leads to clustering of the receptors' death domain[76] (Figure 1). This is supported

by nuclear magnetic resonance structure analysis. An adapter protein called FADD (Fas-associated death domain ; also called Mort1), then binds through its own death domain (DD) to the clustered receptor death domain. FADD also contains a death effector domain (DED) that binds to an analogous domain repeated in tandem within the zymogen form of procaspase - 8 (also call FLICE, or MACH).[77] The death effector domain is a specific example CARD (caspase recruitment domain), which is found in several caspase with large prodomain, including caspse-2,-8,-9,-10 upon recruitment FADD. Caspase-8 oligomerization drives its activation through self-cleavage. Caspase-8 activates downstream effector caspases such as procaspase-9 committing the cell to apoptosis.[67]

In TNF-induced apoptosis, TNF is produced mainly by activated macrophages and T cells in response to infection. In some cell types, TNF also induces apoptosis through tumor necrosis factor receptor 1 (TNFR1) by TNF trimerizes TNFR1 upon binding, including association of the receptor's death domain. An adaptor termed TRADD (TNFR-associated death domain) binds through its domain to the clustered receptor death domains.[77] TNFR1-TRADD complex can bind to FADD to activate procaspase-8, thereby initiating apoptosis.[67] Besides FADD, TNFR1 can engage an adapter called RAIDD (RIP-associated ICH-1/ CED-3 homologous protein with a death domain). RAIDD binds through a death domain of RIP (receptor-interacting protein) and through a CARD motif to a similar sequence in the death effector caspase-2, thereby inducing apoptosis.[79]

The decoy receptor 3 (DR3) shows a closed sequence similarity to TNFR1. It can bind to Apo3L which is related most closely to TNF, and then trigger apoptosis through TRADD and FADD to activate caspase cascade. Apo3L-DR3 and TNF-TNER1 interactions probably have distinct biological roles.[67]

5. The internal signal

The internal signal of apoptosis (due to oxidative stress, irradiation or presence of virus, toxin, chemotherapy drug) surprisingly occurs through the release of cytochome c from the mitiochondria. The permeability of mitochondrial membranes is a critical event that results in the release of cytochome c (a caspase activator), adenosine triphosphate (ATP), Smac/Diablo (a caspase coactivator) , and an apoptosis-inducing factor which activate the nucleases that damage DNA into small fragments.[80-82]

An important question is how mitochondria is able to respond to intracellular signals and release cytochome c. A strong candidate for the signal decoder is a mitochondrial ion channel called mitochondrial permeability transition pore (MPT). MPT appears to be a multisubunit structure that contains the mitochondrial ATP transport and a voltage-dependent anion channel as well as other proteins. It is located at the point where the inner and outer mitochondrial membranes are closely apposed. Presence of oxidants and pathological level of Ca^{2+} cause MTP to open into an irreversible high-conductance state that results in collapse of the mitochondrial membrane potential and release of cytochome c between the two membranes (Figure 2).[71]

A potential proapoptotic transcription factor, TR3 (also called NUR77 or NGFIB), is a known outer mitochondrial membrane protein, normally present in the nucleus, but it can move to mitochondrial surface. TR3 may also induce mitochondrial permeability to induce cytochome c release and apoptosis.[80,81,83] The Bcl-2Bax/Bid family proteins has been reported that it can be translocated to mitochondria (Figure 3). These proteins permeabilize the outer mitochondrial membrane upon interaction with the permeability transition pore complex (for example, Bax) or altenatively, independently of such an interaction (for example, Bid). Intriguingly, it was recently found that p53 moves from the nucleus to the mitochondria where it interact with hsp70 (a heat shock protein), a mitochondria-specific protein. The impact of the interaction between p53 and hsp70 on mitochondrial membrane integrity has not yet been elucidated.[80]

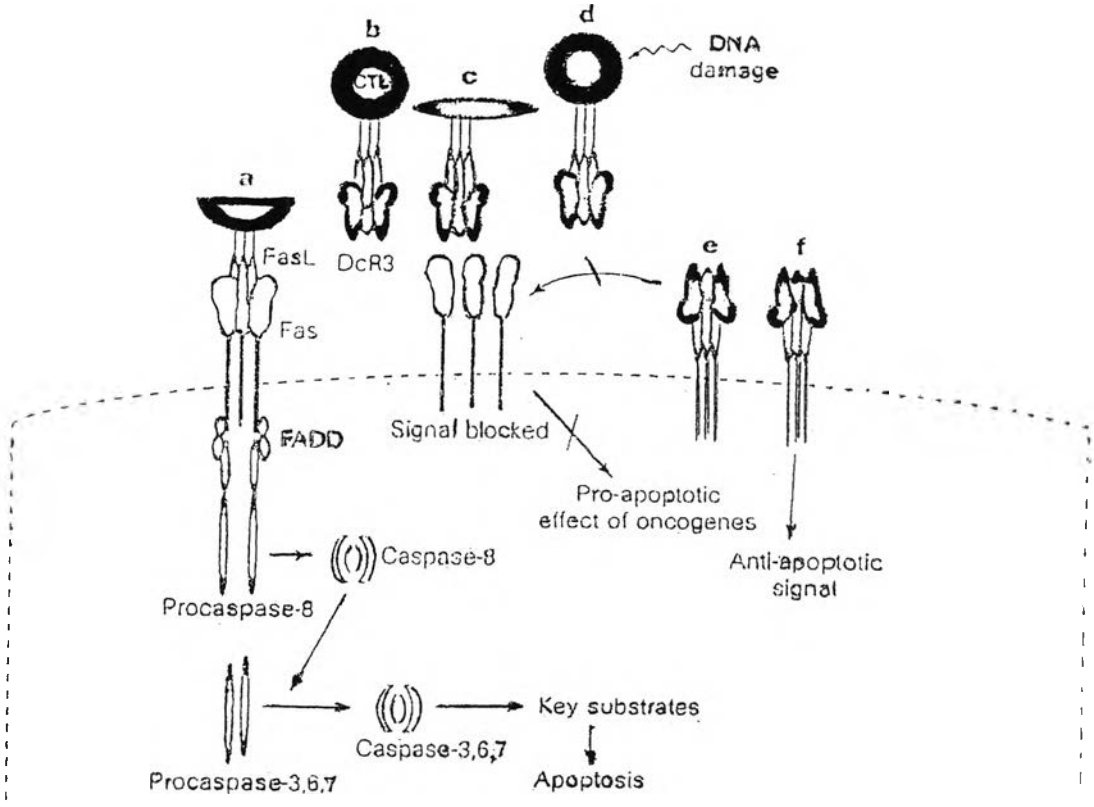

Figure 1 Apoptotic signaling: (a) Normal, trimerization of FasL – bound Fas recruits the cytoplasmic adaptor molecule FADD which, in turn, recruits procaspase-8, the precusor of an intiator caspase (cystein protease with aspartic – acid specific) involved in apoptosis. Two caspase-8 molecules process one another, and assemble to form the mature, active caspase. This cleaves and activates other caspase, which then orchestrate apoptotic cell death. (b-f) Anti-tumorigenesis measure that may be blocked by the binding of DcR3 to FasL. (b) Cytotoxic lymphocytes (CTLs) express FasL. (c) Surrounding cells express FasL in response to tumor infiltration. (d) DNA damage by therapeutic agents induces expression of FasL on tumour cells, which may kill neighboring cells. (e) Fas/FasL signaling normally contributes to the pro-apoptotic effects of oncogenes (such as-c-myc). (f) Ligation of FasL delivers an anti-apoptotic signal to the cell.[73]

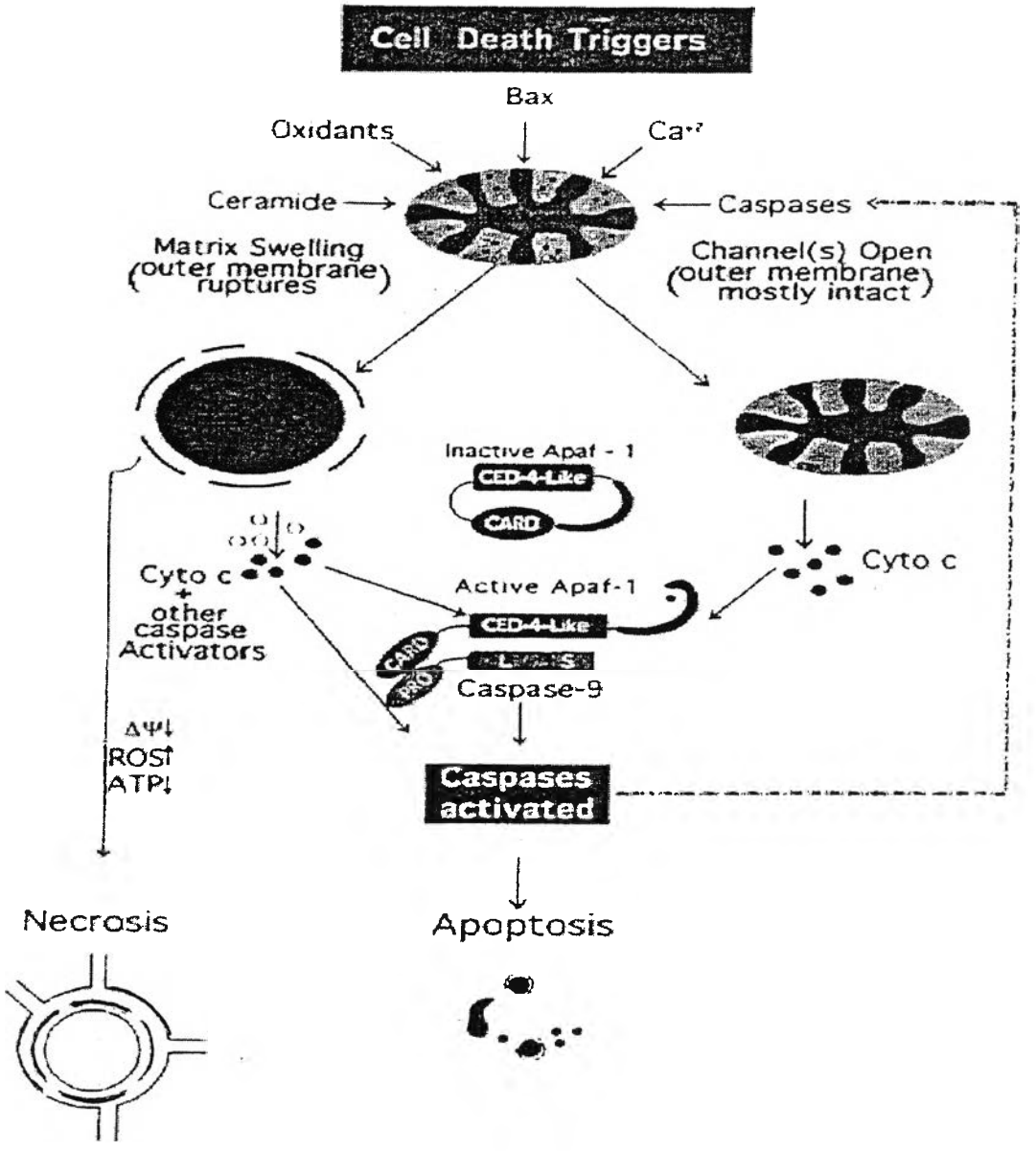

Figure 2 Model for caspase activation by mitochondria. Release of cytochome c into the cytosol results in Apaf-1 activation and subsequent activation of pro-caspase-9 followed by downstream effecting caspases which induce apoptosis.[81]

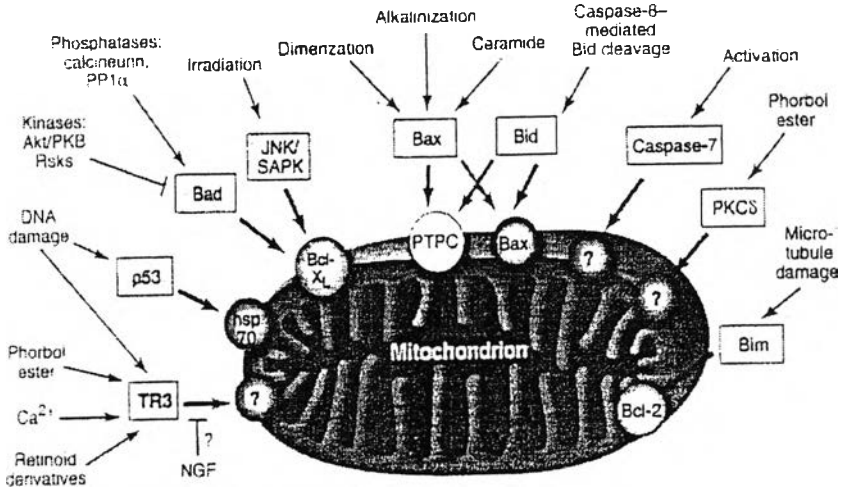

Figure 3 Proteins that move to and affect mitochondrial membranes[80]

In the cytosol, cytochrome c associates with Apaf-1 in the presence of dATP or ATP and induces its oligomerization. The oligomeric Apaf-1 complex recognizes the inactive procaspase-9 and -3, forming the "apoptosome" , which induces autocatalytic processing of procaspase-9. The mature caspase-9 in turn activates its primary downstream target procaspase-3.[66,80]

Whatever the pathway of signaling, the majority of responses occurs through activation of caspase-3. Procaspase-3 can be activated by caspase-8 (external signal pathway) or by caspase-9 (internal signal pathway). In turn caspase-3 can activate other caspase (caspase-6,-9), through proteolytic cleavage at specific internal Asp residues

(Asp-x-x-Asp, where x is any amino acid).[80,84,85] Once activated, the effector caspases are responsible for proteolytic cleavage of a range of cellular targets, ultimately leading to cell death. The summary of trigger apoptosis is shown in Figure 4.

Apoptosis is usually associated with the activation of endonucleases that degrade the chromosomal DNA first into large (50 to 300 kilobases) and subsequently into very small oligonucleosomal fragments.[72,86] Proteolysis of fodrin and actin can account for change on the cell outer surface (blebbing) and that of nuclear lamina for the nuclear fragmentation.[70] During apoptosis, caspases contribute to apoptosis through direct disassembly of cell structure, as illustrated by the destruction of nuclear lamina, which is mainly in the nuclear membrane and is involved in chromatin organization. Lamina is formed by head-to-tail polymers of intermediate filament proteins called lamins. During apoptotic process, lamins are cleaved at a site by caspase, causing lamina to collapse and contributing to chromatin condensation. Caspase also recognizes cell structure indirectly by cleaving several proteins involved in cytoskeleton regulation including gelsolin, focal adhesion kinase (FAK), and p-21 – activated kinase 2 (PAK2).[68]

At the final step of apoptotic process, the asymmetry of plasma membrane phospholipids is lost causing the exposure of PS normally localized at the inner leaflet of the plasma membrance. Appearance of PS on the outer leaflet of the plasma membrane is specifically recognized to uptake and degradation by macrophages and semi-professional phagocytes, which is an important function in the downregulation of inflammatory response after uptake of apoptotic cells by macrophages (Figure 5).[87,88]

6. Clinical aspects of apoptosis

In adult multicellular organism, homeostasis is characterized in each cell lineage by a balance between cell death and cell growth. Dysregulation of cell death mechanism is involved in the pathogenesis of an increasing number of diseases.[89] Defective apoptosis can participate in malignant transformation, viral latency and autoimmune diseases. Excessive apoptotic cell death is involved in CD4+ T-cell depletion observed in acquired immune deficiency syndrome, in fulminant hepatitis associated with infection by hepatitis B and C viruses, in some neurodegenerative disorders and hematological diseases, in polycystic kidney disease and ischemia.[89]

Three steps can be distinguished in the pathway that leads to cell death. The first step involves interaction between the extracellular and intracellular signals that decide whether a cell should die or live. When death is chosen, a common pathway that involves at least the Bcl-2 family of proteins and the interleukin-1-β-converting enzyme-related cysteine proteases continues. Finally, if death is allowed to occur, the apoptotic process itself is characterized by deoxyribonucleic acid (DNA) fragmentation, proteolysis and morphological changes that precede the engulfment of apoptotic cells by neighboring cells and phagocytes.[89]

In clinical aspects, we can divide programmed cell death into 3 main steps, viz. to live or to die, to die or not to die, and to die.

6.1 To live or to die

In adult multicellular organism, different cell types vary widely in the mechanism by which they maintain themselves throughout life. Red blood cells undergo constant renewal from hematological progenitors whereas neurons have no or limited capacity for self-renewal. Between these extremes, lymphocytes and cells from reproductive systems undergo cyclical expansion and contraction as they participate in host defense and reproduction, respectively. Within these cell lineage, the control of cell number is determined by a balance between cell proliferation and cell death.[89]

The mechanism that controls cell proliferation and death could share several factors. Growth factors can either stimulate cell growth or prevent cell death. Antigenic stimulation of T cell receptor first triggers the proliferation of mature peripheral T-cells which are later eliminated by Fas-mediated apoptosis.[90] It depends on cell type and its level of differentiation for the cell to survive or die.[91] Cell survival could depend upon the constant supply of survival factor provided by neighboring cells and extracellular matrix.[92-94]

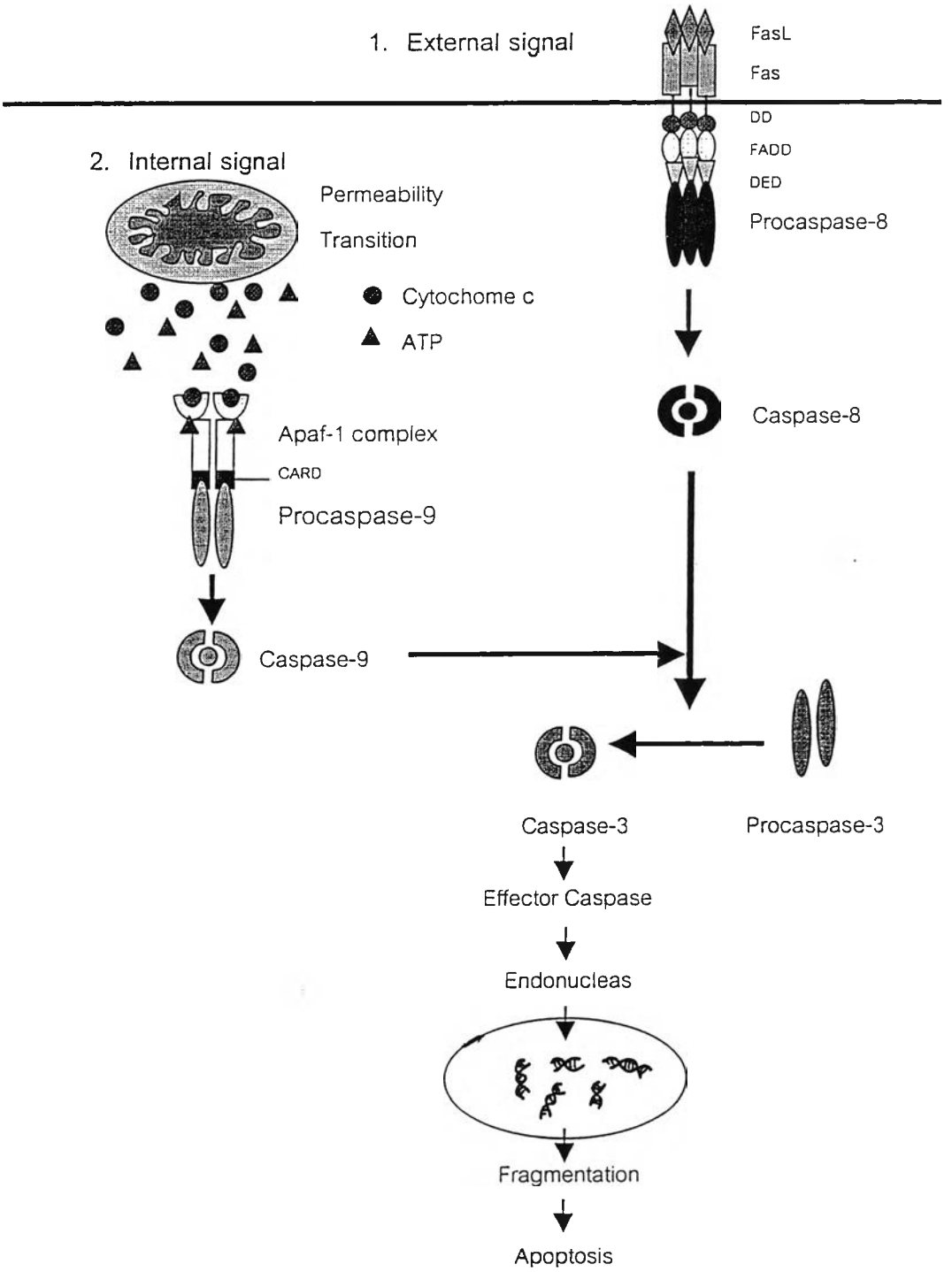

Figure 4 Trigger apoptotic signals

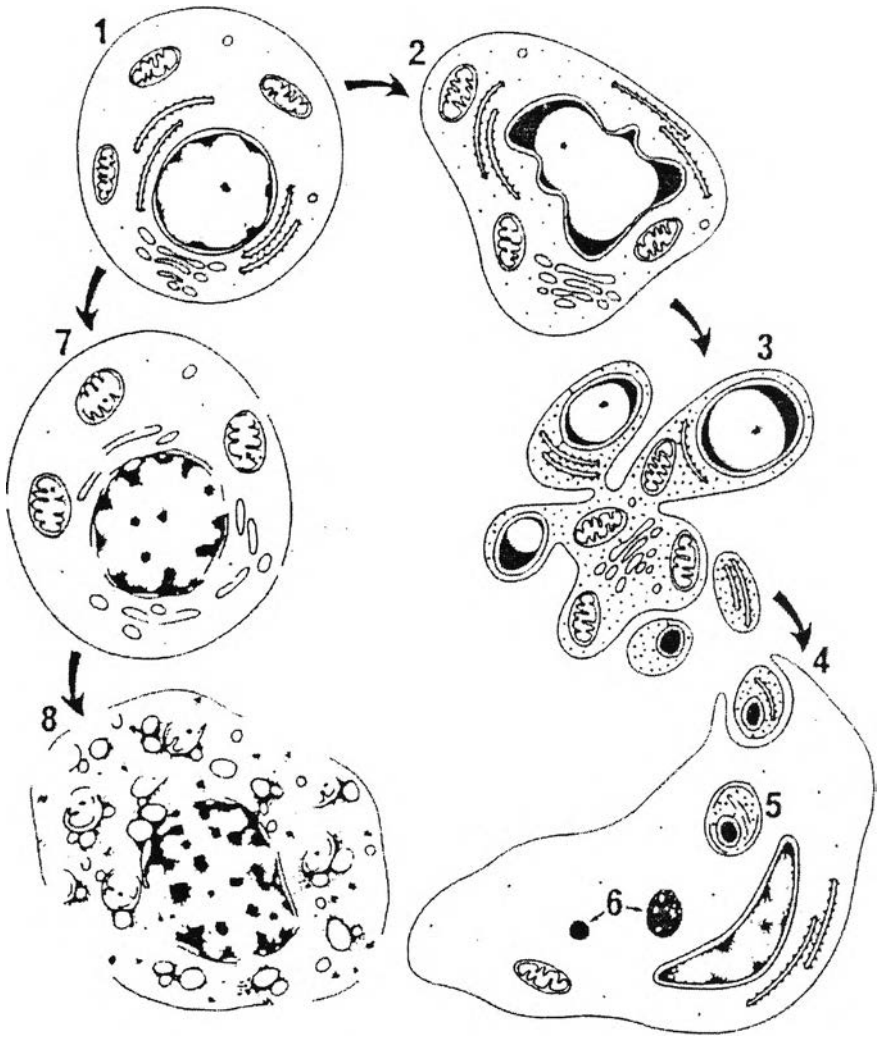

Figure 5 Diagram illustrating sequence of ultrastructural changes in apoptotsis (2-6) and necrosis (7 and 8). (1) Normal cell. Early apoptosis (2) is characterized by compaction and margination of nuclear and chromatin, condensation of cytoplasm, and convolution of nuclear and cell outlines. (3) At the last stage the nuclei fragments and protuberances that form on the cell surface separate into apoptotic bodies, which (4) are phagocytosed by nearby cells and (5 and 6) degraded within lysosome. (7) The development of necrosis is associated with irregular clumping of chromatin, marked swelling of organelles and focal disruption of membranes. (8) Membranes subsequently disintegrate, but the cell usually retains overall shape until removal by mononuclear phagocytes.[86]

6.2 To die or not to die

Beyond different signaling pathways that ultimately converge to activate a cell death signal, apoptosis appears to involve a common final pathway that has been, at least partially, conserved throughout animal evolution. Specific steps that regulate the cell death pathway have been derived from genetic studies of the nematode *Caenorhabditis elegans*.[95] Among the 14 genes whose mutation affects the various steps of programmed cell death, three genes affect the death process itself, namely CED-3, CED-4, that are required for cells that must die to undergo apoptosis, and CED-9 that is required to protect cells that should live from undergoing apoptosis.

Furthermore, the CED-3 gene encodes a protein that is similar to family of cysteine protease, which includes interleukin-1-β -converting enzyme (ICE), Nedd2/Ich-1, CPP32/Yama, Tx/Ich-2 and Mch2.[96] These proteases share a pentameric peptide, QACRG, surrounding a putative activated site Cys. Accordingly, an overexpression of these proteases in mammalian cells causes apoptosis, and the cowpox virus, crmA gene product, inhibits some ICE-like proteases, and can protect mammalian cells against apoptosis induced by growth factor withdrawal.[97] By contrast, the protein encoded by CED-4 gene has no similarity with other known proteins. The protein encoded by CED-9 is homologous to the Bc1-2 family of cell death regulators identified in mammalian cells. Studies performed on the role of cysteine proteases and Bc1-2 related proteins suggest that these two components define two checkpoints in the final common pathway that decides whether or not a cell should die.[89]

6.3 To die

When the final common pathway has allowed the execution of the cell suicide program, characteristic morphological changes of the dying cells[42] precede the production of apoptotic bodies, membrane-enclosed particles containing intracellular material as mentioned. These particles are rapidly engulfed and digested by the neighboring cells and phagocytes to prevent any release of intracellular material that would otherwise trigger an inflammatory response, as observed during necrosis.

Apoptosis is usually associated with the activation of one or several nucleases that degrade nuclear DNA first into large and subsequently into very small fragments. Proteolytic cleavage of several nuclear proteins by an activity similar to but distinct form ICE is another biochemical marker of apoptotic cell death.[98,99]

7. Apoptosis and Disease

In multicellular organisms, homeostasis is maintained through a balance between cell proliferation and cell death. Recent evidences suggest that the failure of cells to undergo apoptosis might be involved in the pathogenesis of a variety of human diseases (Table 2), including cancer, autoimmune disease, viral infections. Moreover, a wide number of diseases characterized by cell loss, such as neurodegenerative disorders, AIDS, and osteoporosis, may result from accelerated rates of physiological cell death.[100,101]

Table. 2 Diseases associated with imbalanced apoptosis[101]

• Diseases associated with the Inhibition of apoptosis	• Diseases associated with the increased apoptosis
1. Cancer	1. AIDS
Follicular lymphomas	2. Neurodegenerative disorders
Carcinomas with p53 mutations	Alzheimer's disease
Hormone-dependent tumors	Parkinson's disease
Breast cancer	Amylotrophic lateral
Prostate cancer	sclerosis
Ovarian cancer	Retinitis pigmentosa
	Cerebellar degeneration
2. Autoimmune disorders	3. Myelodysplastic syndromes
Systemic lupus erythematosus	Aplastic anemia
Immune- mediated	4. Ischemic injury
glomerulonephritis	Myocardial infarction
	Stroke
3. Viral infections	Reperfusion injury
Herpesrviruses	5. Toxin-induced liver disease
Poxviruses	Alcohol
Adenoviruses	

The disease is characterized by the accumulation of cells including cancer, autoimmune disease, and certain viral illnesses. Cell accumulation can result from either increased proliferation or the failure of cells to undergo apoptosis in response to appropriate stimuli. Although much

attention has focused on the potential role of cell proliferation in these diseases, an increasing evidence suggests that alterations in the control of cell survival are important in the pathogenesis of these so-called proliferative disorders.[100-102]

7.1 Apoptosis in cancer

Cancer is envisaged as a disease of excessive cellular proliferation. Genetic alterations that dysregulate the physiological cell death process contribute to the clonal expansion of malignant cells. Accordingly, a number of oncogenes and anti-oncogenes have been found to regulate apoptotic cell death. Oncogenes that promote cell proliferation and those that inhibit cell death could co-operate to induce a neoplastic lesion.

It was confirmed that genetic alteration that induce cell proliferation, e.g., dysregulation of either *myc* or *ras* proto-oncogenes, have been found to be inducers for apoptosis.[58,103] The simultaneous expression of oncogene, e.g., bcl-2, inhibits apoptotic cell death and the transformation is allowed to occur.[61] The best example of these oncogenes is bcl-2, identified at the site of a chromosome translocation between chromosome 14 and 18, present in most human follicular lymphomas.[102] Upregulation of bcl-2 oncogene expression specifically inhibits apoptosis induced by a wide range of insults and stimuli, e.g., growth factor deprivation, loss of contact with extracellular matrix, cytotoxic T cell, cytotoxic lymphokines, chemotherapeutic drugs, and radiation.[104-107] As indicated previously, human bcl-2 can promote cell survival in lower organisms, such as nematodes, and substitute for the loss of function of ced-9.

An elevated level and aberrant pattern of Bcl-2 protein expression have been found in a wide variety of human cancers, including lymphomas, leukemias, adenocarcinomas, neuroblastomas, renal and lung cancers, and melanomas.[104] In most of these tumors, dysregulation of bcl-2 gene expression is not the consequence of (14;18) chromosomal translocation. Structural alterations of the bcl-2 gene are not detected in most leukemias and solid tumors, suggesting that transregulatory rather than cis-regulatory mechanisms account for overexpression of Bcl-2. This overexpression can represent either an early or a late event in the tumor progression.[104]

One of the potential transregulators of bcl-2 that can become altered in cancer is the tumor suppresser p53, which represses bcl-2 gene expression.[108,109] Germ-line p53 mutations predispose individuals with Li-Fraumeni cancer syndrome to the development of tumors and p53 gene becomes inactivated in over half of all human tumors.[110]

The wild-type p53 protein binds DNA and functions, at least, as a transcriptional regulator, activating of repressing the expression of various target genes involved in DNA replication and repair. Wild-type p53 functions primarily to suppress neoplastic growth by inducing apoptosis [111] and contributes to tumor suppression by inducing cell cycle arrest at G1/S checkpoint in response to DNA damage, in order to facilitate DNA repair.[112] The p53 is also an inducer of apoptosis in certain normal tissues, e.g., myeloid progenitors and epithelial stem cells.[113] Its presence or absence is an important factor for the determination of sensitivity of normal and tumor cells to apoptosis inducing by DNA damaging therapeutic agents.

In addition to repressing *bcl-2* gene expression, p53 transactivates the expression of a homologous of *bcl-2* termed *bax*.[108,114] In contrast to Bcl-2 , the protein encoded by *bax* functions as a promoter of cell death. Importantly, Bax and Bcl-2 are members of a large family of proteins that can either promote or repress apoptosis,[115] including Bcl-x, mcl-1, A1, BAK, Bad, and BAG-1.[116-121] These proteins can interact through heterodimerization or homodimerization.[122] The functional significance of these interaction is assumed to regulate the mediators in the pathway of apoptosis as summarized before.

Bcl-x can be expressed under two different isoforms, resulting from alternative of Bcl-x mRNA.[116] Bcl-sL, that functions as a death inhibitor, is markedly devated in several tumors, whereas Bcl-xS could promote cell death by sequestering so that it cannot interact with Bax or other proteins.

7.2 Apoptosis in autoimmune disease

Under normal conditions of the immune system, apoptosis has important roles. For example, apoptosis reduces the autoreaction of T-cells in the thymus that is responsible for self-tolerance and selects B-cells in lymphoid germinal centers during humoral immune response. So a defect in the deletion of autoreactive lymphocytes by apoptosis can predispose to autoimmunity.[100,102] Some evidences in animals and humans indicate that extended survival of autoreactive cells is implicated in at least two chronic autoimmune syndromes: systemic lupus erythematosus and rheumatoid arthritis.[100] Patients with systemic lupus erythematosus have elevated levels of soluble Fas, which may competitively inhibit FasL-Fas interaction. The

resulting decrease in Fas-mediated apoptosis may contribute to the accumulation of autoimmune cells in this disease.[101]

7.3 Apoptosis in viral infection

Cells infected with a virus can undergo apoptosis as a defense mechanism to prevent viral infection. Infected cells can also express viral peptides in association with cell surface major histocompatibolity class 1 molecules, in order to be recognized and killed by cytotoxic T cells. T cells will induce apoptosis, either by using perforin to introduce proteases into the target cell,[123] or by activation of the Fas receptor on its surface. A number of viruses disrupt the normal regulation of apoptosis within infected cells to circumvent the host defense. To reach this goal, viral genes encode inhibitory proteins, most of which target one of the two main checkpoints of the final common pathway that leads to apoptosis. The cowpox virus gene, *crm A*, encodes a protease inhibitor that prevents apoptosis by specifically inhibiting ICE, a key protease in the final pathway of Fas and tumor necrosis-α-mediated cell death.

Other viral genes encode a protein with structural and functional similarities with *Bcl-2* including *BHRF-1* gene of Epstein Barr virus, the *LMW5-HL* gene of African swine fever virus, and the *E1B* gene of adenovirus.[104,124,125] The product of some viral gene, e.g., LMP-1 from Ebstein-Barr virus, can upregulate *Bcl-2* to allow the establishment of viral latency.[126]

The mechanism by which the *p53* gene, identified in baculovirus as a potent inhibitor of apoptosis, inhibits cell death in infected cells is still not understood. Intriguingly, another baculovirus gene that inhibits apoptosis, namely inhibitor of apoptosis protein (IAP), is analogous to a gene involved in the pathogenesis of a recessive neurodegenerative disorder observed in children.[126] Although biochemical mechanisms by which viral proteins inhibit cell death remain poorly explained, nitric oxide produced by human B-lymphocytes was recently reported to contribute to the maintenance of viral latency in down regulating the expression of the Epstein Barr virus early antigen.[128]

7.4 Apoptosis in AIDS

AIDS is characterized by a progressive and selective depletion of the $CD4^+$ population of T-lymphocytes. The exact mechanisms by which the human immune deficiency virus-1 (HIV-1) kills immune cells is not understood. Most T- cells that die during HIV infection do not appear to be infected with virus and the number of apoptotic T-cells does not correlate with progression of disease.[129] Nevertheless, a growing body of experimental evidence suggests a role for apoptosis in $CD4^+$ T-cell depletion. Enhanced apoptosis has been observed in primate models of lentiviral infections, as well as in lymphocytes and lymph nodes from AIDS patients. Picomolar concentration of soluble viral product, gp120, were reported to prime human $CD4^+$ T-cells for activation-induced cell death.[130] More recently, HIV-1 Tat protein was shown to induce cell death by apoptosis in T-cell lines and in mononuclear peripheral blood cells from uninfected donors.[131] Tat protein was shown to induce in T-cells a

premature activation of cyclin-dependent kinases, an event that has been associated with apoptosis induction in several other cell systems.[89]

Fas could be involved in the death of $CD4^+$ T-cells during the course of an HIV infection.[132] Human T-cell lines, transformed with HIV, are more sensitive to Fas mediated apoptosis than parental cells. Fas is highly expressed on T-cells of mice with retrovirus-induced immunodeficiency syndrome and on T-lymphocytes of HIV-infected children. The current hypothesis is that HIV-1 Tat and gp120 accelerate Fas mediated, activation-induced T-cells apoptosis, therefore contributing to $CD4^+$ T-cell depletion during the course of AIDS.[131] Lymphocyte apoptosis has also recently been involved in the pathogenesis of leukemia-like disease induced by the human T-lymphocytes virus-1 (HTLV-1) infected T-cells and virally-induced abortive activation of T-cells may be a response to several other viral infection in mammals.[133]

7.5 Hematological diseases

Hematopoietic growth facors, including stem cell growth factor, colony-stimulating factors, erythropoietin and thrombopoietin, play a key role in the regulation of hematopoiesis. These factors were demonstrated to act, in part, by promoting the survival of progenitor cells, by suppressing apoptosis during the differentiation of intrinsically committed progenitors.[134,135] Overexpression of *Bcl-2* prevents apoptosis of hematopoietic cells induced by growth factor withdrawal.[136] Hematological diseases, such as myelodysplastic syndromes, aplastic anemia, chronic neutropenia or severe β-thalassemia are associated with increased apoptotic cell death within the bone marrow. The mechanisms by which increased

apoptotic cell death is involved in the etiology of these diseases remains unexplained and could involve stroma cell deficiencies, gene deregulation and direct effects of toxins or mediators of the immune response.[89]

7.6 Apoptosis in neurodegenerative disorders

The same mechanisms could apply for the increased apoptosis of specific sets of neural cells described in several neurological diseases such as Alzheimer's and Parkinson's diseases, or cerebellar degeneration. The hematopoietic growth factors, several apecific and less specific growth factors, and extracellular matrix prevent neural cell apoptosis, an effect that can be mimicked by overexpression of *Bcl-2* in the neural cells. Several gene mutations that lead to increased apoptotic cell death were identified in neurodegenerative disorders. Mutation in a superoxide dismutase gene were identified in patients with autosomal dominant amyotrophic lateral sclerosis.[137] These mutations decrease the ability of motor neurons to detoxify oxygen-free radicals. Mutations in any of the three photoreceptor-specific genes lead to photoreceptor apoptosis and retinal degeneration observed in patients with retinal pigmentosa.[138] Either dysfunction of the mutated protein or its accumulation could be responsible for increased apoptosis. Mutations in the neuronal apoptosis inhibitory protein (NAIP) gene, a gene homologous to IAP from baculovirus, have been identified in spinal muscular atrophy and may decrease the apoptotic threshold of spinal cord neurons.[127]

8. Methods for the detection of apoptosis

Numerous methods of induced apoptosis are used for the induction of apoptosis *in vitro* for scientific studies. There are a number of laboratory procedures available for detecting and quantifying levels of apoptosis in cell cultures. The simplest of these is an examination of the morphology of cells that are stained with dyes such as heamatoxylin and eosin. [139,140]

An alternative method that is widely used is extraction and analysis of DNA on agarose electrophoresis gels to detect internucleosomal DNA fragmentation. The DNA samples were electrophoretically separated in 1% agarose gel containing ethidium bromide. DNA was visualized with a UV transilluminator, and the gels were photographed. The tell-tale ladder pattern is an indicator of cell death via apoptosis.[141-143] The degree of DNA fragmentation that occurs during the apoptotic process is sensitive for detect apoptotic cells via method, such as, the *in situ* terminal deoxynucleotidyl transferase (TdT) assay, using digoxigenin labeled nucleotides by TdT reaction at the $3'$-OH end of the DNA fragmentation. The incorporated digoxigenin-nucleotides within the cells were detected with fluorescein labeled antidigoxigenin antibodies. The antidigoxigenin antibodies fragment carries a fluorophore (fluorescein) to the reaction site. When excited by light of 494 nm wavelength, the fluorescein generates an intense signal at 523 nm. Finally, the cells were counter stained with 4,6-diamino-2-phenylindole (DAPI). DAPI will stain the nuclei and these fluorescein labeled apoptotic nuclei were detected by fluorescence microscope (Figure 6).[141-145]

The fluorescence analysis of DNA unwinding (FADU) assay has been used to measure DNA single strand breaks, DNA double strand break, alkali-labelled lesion and detected DNA fragmentation which associated with apoptotic nuclei. When double-strand DNA is exposed to moderately alkaline solutions, hydrogen bonds are broken and the 2 strands are unwond. It has been observed that the rate of unwinding of DNA fragments in alkali is increased by prior exposure of cells of DNA unwinding which can be used as a sensitive measure of strand break.[145,146]

Comet assay or the single-cell gel electrophoresis is a simple, rapid and inexpensive method for DNA strand break detection in individual cell. Because apoptosis is characterized by extensive DNA cleavage, this assay has proved to be useful in detecting apoptotic cells as those in which only a small amount of DNA stays in the original position of the nucleus.[147]

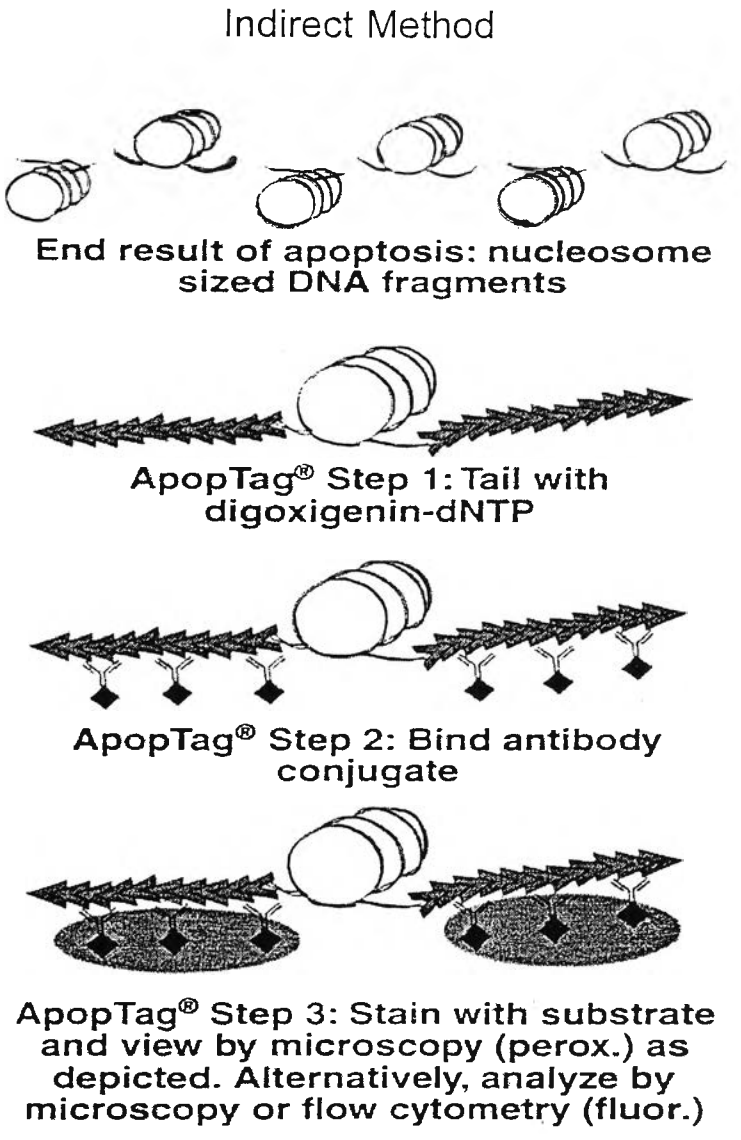

Figure 6 Process of *in situ* terminal deoxynucleotidyl transferase (TdT) assay.[24]

The lymphocyte system

Lymphocytes, the cells that are vital to the immune system, are derived from stem cells through lymphoid lineage. They account for 20 to 40 % of the entire circulating white blood cells. Lymphocytes comprises of T cells (65 to 75 %), B cells (10 to 15 %) and NK cells (5 to 15 %). In addition to appearing in circulating lymphocytes, they are also found in the lymphoid organs such as thymus, spleen, lymph node and the like.[148]

T lymphocytes

Stem cells in the yolk sac, the liver, the spleen or the bone marrow will transform into precursor of T lymphocytes called pre-T cells and travel to the thymus, one of the primary lymphoid organs. In the thymus, pre-T cells will transform into thymocytes. These cells will proliferate and then travel from the thymus to the peripheral lymphoid organs, such as the lymph node, the spleen, etc., where mature lymphocytes transit to the blood. The lymphocytes derived from stem cells via the thymus are termed T-lymphocytes or T-cell (T stands for thymus derived or thymus dependent). They function in the Cell Mediated Immunity (CMI)-type Acquired. T-lymphocytes in the body can be classified into various types according to function:

1. Regulatory cell is the T lymphocyte that functions in controlling the humoral immune system. It controls the functions of the T and B lymphocytes. There are two types of regulatory cell:
    1.1 Helper T cell is the T lymphocyte that promotes the functions of other B and T lymphocytes.

1.2 Suppressor T cell is the T lymphocyte that decreases the function of other lymphocytes.

2. Lymphokine-producing T lymphocyte is responsible for CMI, i.e. the production and the release of lymphokines.
3. Cytotoxic or Killer T cell is the T lymphocyte that is capable of destroying other cells, for example, the destruction of foreign cells.
4. T lymphocyte that is responsible for transplantation immunity like graft rejection. [148-150]

B lymphocytes

The B lymphocyte is another class of lymphocytes derived from stem cells. In the study of poultry, it was found that stem cells in the yolk sac travel to bursa of fabricius, a tiny organ next to the large intestine near cloaca. The bursa of fabricius influences the stem cells to transform and proliferate into a large number of mature lymphocytes and then transit to the lymph nodes, the spleen or the blood. The lymphocyte derived from bursa of fabricius is termed B lymphocyte or B cell (B stands for bursal derived or bursal dependent).[148-150]

In the mammal, an organ functions for the development of B lymphocytes is thought to be the liver in the fetus or the bone marrow in the adult. These B lymphocytes are responsible for Humoral Mediated Immunity (HMI)-type Acquired immune mechanism.[148-150]

Lymphocyte recirculation

The majority of lymphocytes presented in the blood, the tissue, the lymph nodes and the organs are generally circulated all the time. The lymphocytes in the peripheral blood will enter the lymphoid tissue, pass through endothelial cells of capillary venues via diapedesis process. Some of those lymphocytes will exist in the tissue. The remaining lymphocytes will move to lymphatic circulation, enter the lymph node via afferent lymphatic vessel, pass through cortex and medulla, and exit via efferent lymphatic vessels. These lymphocytes then travel via thoracic duct to circulate in the heart and recirculate to the blood via the artery flowing to several organs, for instance, the lung, the spleen, the liver and the cutaneous tissues. [148-150]

The lymphocyte recirculation requires the transmission of data among the blood system, the lymph and the area where antigen flowing with lymphoid tissues for the condition of immune response. [148-150]

There are two classes of circulating lymphocytes: native T cells ($CD45RA^+$) that directly enter the lymph nodes, and memory T cells ($CD45RO^+$) that usually travel to the areas where antigen appears and the inflamed areas around the tissues like mucosal lymphoid tissue and Peyer's patches, etc. These effector cells which are responsible for the immune response are short-lived with a life span of about 2-4 days to 1-2 weeks.[150]

Unilateral ureteral obstruction (UUO) and immune cell

The study of immune cell features subjected to UUO[12] found that there is an increased influx of leukocytes within the obstructed kidney. A leukocyte influx, predominantly macrophages and T lymphocytes; either cytotoxic T cells or suppressor T lymphocytes, occur in both the cortex and medulla. An increase in leukocyte infiltration is apparent as early as 4 hours and is at the maximum level after 24 hours of obstruction, increasing 10-fold higher than normal. In addition, it is also found that there is an increase in helper cell subpopulation of circulating T lymphocytes.[12]

A mechanism of the infiltration of macrophages and lymphocytes within the kidney tissue results from increasing levels of Ang II in UUO.[13] Ang II stimulates the expression of monocyte chemoattractant peptide-1 (MCP-1) and osteopontin growths.[151] Both MCP-1 and osteopontin induce inflammatory cells, particularly macrophages and lymphocytes in the kidney and these cells bind to tubular and endothelial cells with adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1). Macrophages and lymphocytes derive cytokines, such as transforming growth factor- β (TGF-β), up-regulated directly by Ang II, that plays a pivotal role in the progression of renal diseases.[152-156] TGF- β inhibits cell growth and stimulates matrix regeneration by increasing the synthesis of matrix receptors like integrins and osteopontin. In addition, TGF- β inhibits matrix degradation by increasing the activity of tissue inhibitors of metalloproteinases (TIMP) and by decreasing the activity of metalloproteinases resulting in the expansion of matrix and interstitial fibrosis. Furthermore, TGF- β is a chemoattractant for fibroblasts and stimulates fibroblasts proliferation.[157,158] According to

the studies, activation of TGF- β is attenuated when angiotensin 1 receptor antagonist (ARA) or when angiotensin converting enzyme inhibitor (ACEI) are used.[154,157-161]

UUO and apoptosis

UUO is a condition that causes remarkable changes, i.e. tubular cell apoptosis. This probably accounts for a renal tissue loss[158,162-164] which ultimately reflects the imbalance of cell proliferation and cell death. UUO somehow elevates the levels of Ang II that promotes activation of ROS and decreases process of antioxidant[158,165] in the body. ROS elevate activation of endonucleases which in turn result in tubular cell apoptosis.[166]

The preliminary studies of a role for Ang II showed that when ACEI were used, enalapril decreased tubular cell apoptosis and partially prevented the renal tissue loss in the early phrases of UUO.[167] In addition, Morrissey et al. reported that AT_2 antagonist did not improve interstitial fibrosis in kidney with UUO, but might blunt or delay tubular cell apoptosis by decreasing p53 expression.[168] p53 is a tumor suppressor gene. The main function of which in the arrest of cell cycle between G1 and S phrases is to activate p21, an inhibitor of G1-cyclin/CDK and PCNA (proliferating cell nuclear antigen). Besides, p53 also activates GADD45 (growth arrest and DNA damage 45) which plays a role in the G1 arrest of cell cycle to repair damaged DNA before the ongoing progression. If DNA repair fails, it may then lead to cell apoptosis.[169] Morrissey et al. employed reverse transcriptase polymerase chain reaction and reported a 13-fold increase in the level of p53 Mrna in 5 days after UUO.[170] In another study by Cummings, measuring mRNA directly on Northern blots, a constant

2.5-fold increase in p53 mRNA level in the obstructed kidney was observed from day 2 throughout day 14. This increase was associated with a progressive increase of tubular cell apoptosis. [171]

In 1997, Ma et al. studied kidneys of AT_2 receptor null mutant mice with UUO and noted the same degree of interstitial fibrosis improvement, but much less tubular cell apoptosis.[172] A report by Chevalier et al. suggested that Ang II stimulated renal cell proliferation through AT_1 receptor in kidneys of both neonatal and adult rats with UUO, but induced tubular cell apoptosis only in adults.[173] However, the mechanism of Ang II remains unclear.

T lymphocytes mediate not only TGF- β but also some cytokines. The major cytokine is TNF comprising TNF- β and TNF-α. TNF- β features destroy as well as stimulate apoptosis process. On the other hand, TNF- β also could promote the growth of cells such as fibroblast and activated B lymphocytes.[174] While TNF-α has a wide spectrum of consequences on cells and systems by stimulating endothelial cells of blood vessel to display adhesion molecules at the surface. This is suitable for the leukocytes, particularly neutrophils and monocytes, to bind and transmiss from the vessel to contaminated tissues. Moreover, TNF-α also stimulates mononuclear phagocytes like monocytes and macrophages that can synthesize other cytokines such as IL-1, IL-6, TNF-α, chemokines, etc. TNF-α leads to cell apoptosis by initially binding to receptors, signaling internally through Fadd to caspase enzymes and then stimulating transcriptional factors that induce apoptotic genes such as Bax, p53 and so on[167], which eventually contribute to the progression of apoptosis.

The studies have demonstrated that lymphocytes have a relevant function to immune system. That is, B lymphocyte cells induce antibody or immunoglobulin in humoral immune response stimulating by antigen and other cells, such as T lymphocytes and macrophage. Aside from stimulating B lymphocytes to induce antibody, T lymphocytes also operate as cell mediated immune response like cytotoxic T cell whose function is to eradicate target cells, null cells (non-B non-T cells) or natural killer cells whose function is to destroy foreign stuff or cancer cells by flowing natural killer cytotoxic factor (NKCF), etc.[174]

In a study of chronic renal failure[17] in rats with increasing levels of Ang II and ROS, it was found that DNA damage of circulating lymphocytes increased the progression of lymphocyte apoptosis and ARA could inhibit such DNA damage.[17] Furthermore, when supplied ACEI to rats with chronic renal failure, the level of antioxidant enzymes increased.[18] To date, there is no study of UUO related to circulating lymphocyte apoptosis as well as the role of angiotensin system on this regard.