CHAPTER 1

INTRODUCTION
1.1 Apoptosis: An overview

Programmed cell death or apoptosis is a genetically programmed, highly conserved, intricate mechanism of cellular suicide, that can be triggered by a variety of physiological and pathophysiological conditions and which plays a crucial role in the development and maintenance of cellular homeostasis in multicellular organisms (reviewed by Arends and Wyllie, 1991; Wyllie et al., 1980). Apoptosis is essential for normal development and deregulation of the process leads to a spectrum of defects ranging from embryonic lethality, tissue specific perturbation of post-natal development, and a high susceptibility to cancer (reviewed by Thompson, 1995). Apoptosis is invoked in a wide variety of different biological systems, including normal cell turnover, development of the immune system, embryonic development, metamorphosis, growth factor withdrawal, exposure to chemotoxins or even physical damage (reviewed by Ellis et al., 1991; Cohen et al., 1992b). Apoptotic cell death is characterized by a series of distinct morphological and biochemical alterations which include cell shrinkage, membrane blebbing, chromatin condensation and fragmentation, formation of apoptotic bodies that are rapidly devoured by phagocytes (reviewed by Thompson, 1995).

1.2 Historical perspective

The phenomenon of cell death occurring as a normal part of both development and homeostasis was established more than forty years ago (reviewed by Glücksman, 1950). In vertebrates, cell death has been observed in almost all tissues and has been studied more extensively in the developing nervous system (reviewed by Hamburger and Oppenheim, 1982) and in the immune system (reviewed by Duvall and Wyllie, 1986).

In the early 1970s, Kerr, Wyllie & Curie found that cells undergo at least two distinct forms of death: the well characterized and usually necrotic tissue damage induced by trauma,
and a more protracted and morphologically distinct form of cell death which they termed 'apoptosis' (reviewed by Kerr et al., 1972; Wyllie et al., 1980). Apoptosis in archaic Greek refers to leaves falling off trees in the autumn. The word was chosen to suggest cell loss that is desirable for the survival of the host (reviewed by Touchette and Fogle, 1991). On the contrary, the term 'programmed cell death' (PCD) was used to describe cell death that occurred in predictable places and at predictable times during development, to emphasize that the death is somewhat programmed into the developmental plan of the organism. Using this strict definition, cell death during cyclic or seasonal involution of gonads, turnover of epithelia and hematopoietic cells, morphogenesis in embryonic development, metamorphosis, and a few other examples is clearly programmed. While the term 'apoptosis' etymologically evokes a programmed occurrence in the organism, the current tendency is to use it in reference to the morphological changes accompanying this type of cell death which includes cytoplasmic and nuclear condensation / fragmentation. There are examples of truly programmed cell death that is not apoptosis by morphology (reviewed by Schwartz and Osborne, 1993). But often both these terms are used interchangeably.

The existence of an intrinsic cell suicide program was ascertained through genetic studies carried out by Horvitz and colleagues in the nematode *Caenorhabditis elegans* that identified genes involved in the cell death program and its control (reviewed by Ellis and Horvitz, 1986; Horvitz and Ellis, 1982), and then found out that some of these genes were homologous to mammalian genes (Hengartner and Horvitz, 1994; Yuan et al, 1993). These studies demonstrated the very early occurrence of PCD in the course of metazoan evolution and the substantial conservation of its basal machinery from nematodes to humans.
1.3 Characteristic features of apoptotic cells

1.3.1 Morphological changes associated with apoptosis

Apoptosis is characterized by a distinctive and orchestrated sequence of morphological changes. In the initial phase, an individual cell embedded in normal tissue loses contact with its neighbors (reviewed by Wyllie et al, 1980). The cell shrinks due to loss of cytoplasmic volume (shrinkage necrosis) and condensation of cytoplasmic proteins. The nuclear chromatin gets condensed resulting in the fragmentation of cellular DNA. The second phase is characterized by membrane ruffling and blebbing, leading to cellular fragmentation and formation of apoptotic bodies, frequently containing nuclear remnants (Williams et al., 1974; Wyllie, 1980; reviewed by Arends and Wyllie, 1991). While most of these events are taking place, the cell still excludes vital dyes and retains most of its internal constituents. In the final phase, the neighboring cells and macrophages phagocytose the fragments for complete degradation.

1.3.2 DNA degradation in apoptosis

Induction of apoptosis is characterized by the internucleosomal DNA cleavage, a biochemical event used as a definitive apoptotic marker (reviewed by Schwartzman and Cidlowski, 1993). This pattern of DNA degradation occurs by activation of an endogenous endonuclease that cleaves the DNA in the linker region between histones on the chromosomes. Since the DNA wrapped around the histones comprises ~180-200 bp, multiples of this fragment are characteristicly observed and are commonly referred to as the 'apoptotic ladder' (Wyllie, 1980; reviewed Kerr et al., 1972). Despite overwhelming evidence in favour of internucleosomal DNA cleavage activity, as a characteristic of apoptosis, several accounts of apoptosis in the absence of this DNA cleavage pattern have been reported (Zakeri
et al., 1993; Boe et al., 1991; Oberhammer et al., 1992). Cleavage of DNA into large fragments of 50-300 kb during apoptosis have also been reported (Walker et al., 1991).

One of the main nucleases involved in apoptotic DNA degradation is caspase-activated DNase (CAD) (Enari et al., 1998; Liu et al., 1997). CAD is normally complexed to its chaperone and inhibitor, ICAD. In dying cells, ICAD is cleaved by caspases, releasing the inhibition on CAD. However, CAD-deficient cells fail to undergo oligonucleosomal DNA fragmentation, if stimulated to undergo apoptosis (Liu et al., 1998) but still exhibit other features of apoptotic cell death. The sequence of murine ICAD is highly homologous to a human protein, DFF45 which also contains caspase-3 sites and is cleaved during apoptosis (Liu et al., 1997). DNA degradation may serve to limit potentially dangerous genetic information spreading from the dying cells and also to avoid persistence of auto-antigenic material such as nucleosomes which may be involved in the pathogenesis of auto-immune disorders.

1.3.3 Phagocytosis of apoptotic cells

One of the striking features of apoptotic cell death is the rapid engulfment of the dying cells by phagocytes and the complete elimination of cell corpses, thus protecting the surrounding tissue from the damaging effects of released intracellular contents. Phagocytosis is triggered by physiochemical changes in the plasma membrane of the dying cells that function as "eat me" signals, enabling the heterophagic recognition and engulfment of the apoptotic cells by adjacent healthy cells. One of the well-characterized signals for phagocyte recognition of apoptotic cells is the loss in plasma membrane phospholipid asymmetry (reviewed by Engeland et al., 1998; Fadok et al., 1998). Viable cells maintain an asymmetric distribution of different phospholipids with phosphatidyl choline and sphingomyelin being
present on the outer leaflet, and phosphatidylethanolamine and phosphatidylserine (PS), restricted to the inner leaflet of the membrane.

It has been demonstrated that cells undergoing apoptosis expose PS on the outer leaflet of the plasma membrane (Fadok et al., 1992). This cell surface exposed PS, functions as a tag for specific recognition by macrophages and for phagocytosis of the dying cell. This phenomenon has shown to be an early event during apoptosis, initiated at a time following the caspase proteolytic cascade but possibly preceding nuclear condensation and breakdown of intracellular cytoskeletal and nuclear matrix constituents (Martin et al., 1995b).

1.4 Caspases: The executioners of apoptosis

Caspases (Cysteiny1 aspartate-specific proteinases) mediate highly specific proteolytic cleavage events in dying cells which collectively manifest the apoptotic phenotype (reviewed by Alnemri et al, 1996). The key and central role that these enzymes play in a biochemical cell-suicide pathway has been conserved throughout the evolution of multicellular organisms. Caspases were implicated in apoptosis with the discovery that CED-3, the product of a gene required for cell death in the nematode Caenorhabditis elegans, is related to mammalian interleukin 1β-converting enzyme (ICE or caspase-1) (Yuan et al., 1993; Thornberry et al., 1992). More than a dozen caspases have been identified in humans, about two-thirds of these have been suggested to function in apoptosis (reviewed by Earnshaw et al., 1999; Thornberry and Lazebnik, 1998). The current nomenclature as well as the trivial names of some of the caspases is shown in Table 1.1.

1.4.1 Structure, activation and regulation of caspases

Caspases are synthesized as inactive pro-enzymes which are activated by cleavage adjacent to aspartate residues to liberate one large and one small subunit, which associate into
an $\alpha_2\beta_2$ tetramer to form the active enzyme. The absolute requirement for cleavage next to aspartate enables caspases to activate other caspases, thereby setting the stage for an amplifying cascade. Recognition of at least four amino acids NH$_2$-terminal to the cleavage site is also a necessary requirement for efficient catalysis. The preferred tetrapeptide recognition motif differs significantly among caspases and explains the diversity of their biological functions (Thornberry et al., 1997).
<table>
<thead>
<tr>
<th>Current name</th>
<th>Trivial name</th>
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<tbody>
<tr>
<td>Caspase-1</td>
<td>ICE</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>Ich-1L, NEDD2</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>CPP32, Apopain, Yama</td>
</tr>
<tr>
<td>Caspase-4</td>
<td>TX Ich-2, ICE rel-II</td>
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<tr>
<td>Caspase-5</td>
<td>ICE rel-III, TY</td>
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<tr>
<td>Caspase-6</td>
<td>Mch 2</td>
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<tr>
<td>Caspase-7</td>
<td>Mch 3, CMH-1, ICE-LAP3</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Mch5, MACH, FLICE</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>Mch6, ICE-LAP6</td>
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<tr>
<td>Caspase-10</td>
<td>Mch4, FLICE-2</td>
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<tr>
<td>Caspase-11</td>
<td>mlCH-3, mCASP-11</td>
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<tr>
<td>Caspase-12</td>
<td>mlCH-4, mCASP-12</td>
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<td>Caspase-13</td>
<td>ERICE</td>
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<td>Caspase-14</td>
<td>MICE</td>
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Schematic representation of the general pathway of apoptosis
Caspases may be divided into two classes based on the lengths of their N-terminal prodomains. Caspase-1, -2, -4, -5, -8, 10 have long prodomains; whereas caspases-3, -6, -7 and -9 have short prodomains.

1.4.2 Cellular targets of caspases

The role of proteases in cell suicide is to disable critical homeostatic and repair processes, as well as to cleave key structural components, resulting in the systematic and orderly disassembly of the dying cell. Several important caspase substrates have been identified in recent years. Polypeptides known to be cleaved during apoptosis include enzymes involved in genome function such as poly (ADP-ribose) polymerase (Lazebnik, et al., 1994), the catalytic subunit of DNA-dependent protein kinase (Emoto et al., 1995), the 70-kDa protein component of the U1 small ribonucleoprotein (Tewari et al., 1995), ICAD (Sakahira et al., 1998) etc. Cleavage of nuclear lamins is required for nuclear shrinkage and budding (Rao et al., 1996). Loss of overall cell shape is probably caused by the cleavage of cytoskeletal proteins such as fodrin and gelsolin (Kothakota et al., 1997), P21-activated kinase-2 (PAK-2) (Rudel and Bokoch, 1997) and many more. Caspases thus help to cut off contacts with surrounding cells, reorganize the cytoskeleton, shut down DNA replication and repair, disrupt the nuclear structure and disintegrate the cells into apoptotic bodies.

1.4.3 Inhibitors of caspases

Several classes of natural as well as synthetic caspase inhibitors have been described that can subvert the cell suicide program. These include
(i) **Bcl-2 family of proteins**

Bcl-2 was first isolated as the proto-oncogene involved in t(14; 18) translocation found in human follicular lymphomas (Bakshi et al., 1985). Bcl-2 and related family members, such as Bcl-X\(_L\), inhibit cell death induced by many stimuli (Vaux et al., 1988). Several recent studies suggest that Bcl-2 and Bcl-X\(_L\) exert their anti-apoptotic action at or before the processing of certain caspases to their catalytically active forms (reviewed by Chinnaiyan et al., 1996a). The function of Bcl-2 is described in more detail in section 1.5.4.

(ii) **CrmA, p35, cellular IAP’s**

These are the naturally occurring caspase inhibitors. Cytokine response modifier (CrmA) is a 38 kDa serpin from Coxsphox virus that appears to facilitate viral infection through both inhibition of the host inflammatory response and inhibition of apoptosis (Ray et al., 1992). CrmA has been evaluated as an inhibitor of both caspase-1 and caspase-8 (Nicholson et al., 1995).

p35, a 35-kDa protein from Baculovirus also appears to attenuate apoptosis through inhibition of caspases (Xue and Horvitz, 1995). Synthesis of p35 has been shown to prevent cell death in insect cells, in *C. elegans* and in mammalian systems.

The IAP (inhibitor of apoptosis) gene family, comprise a third group of polypeptides that prevent cell death in a variety of species. One family member, XIAP, has been shown to be a direct inhibitor of caspase-3 and -7 (Deveraux et al., 1997)

(iii) **Synthetic peptide inhibitors**

Synthetic peptide inhibitors that mimic cleavage sites of the caspases (usually at an Asp-X bond) have been used for *in vitro* and *in vivo* analysis of enzyme activity (Villa et al.,
These peptides are generally very small, 3 to 4 amino acids in length and include zVAD-fmk (broad spectrum inhibitor), YVAD-cmk (caspase-1 inhibitor), DEVD-CHO (caspase-3 inhibitor) etc. The sequence of these various peptides are based on the caspase recognition sites of substrates. In zVAD-fmk, due to an aspartate residue mimicking the cleavage site and a fmk group, forming a covalent inhibitor/enzyme complex, the inhibitor instantly and irreversibly binds to the catalytic site of caspases (Garcia-Calvo et al., 1998). These peptide inhibitors are soluble, relatively stable and show dose-dependent inhibition of cell death after induction with several different stimuli. These inhibitors can be used to block individual caspase proteases, allowing the definitive identification of each protease involved in the processing of specific substrates, as well as helping to order the upstream and downstream events within a particular apoptotic signaling pathway.

1.4.4 Genetic analysis of the biological role of caspases

The role of individual caspase in apoptotic process has been deciphered by the targeted disruption of these enzymes in mice. It was seen that no caspase knockout described till date abolishes all apoptosis during development and for any caspase, defects in apoptosis are both cell type and stimulus-dependent.

(i) Caspase-1

ICE/caspase-1 is the cysteine protease responsible for the proteolytic conversion of the 31 kDa inactive cytokine precursor, pro-interleukin 1β, to its 17.5 kDa active form, a key mediator of inflammation (Kostura et al., 1989). Caspase-1 was shown to share 28% sequence identity to C. elegans CED-3 and shown to play a role in apoptosis (Yuan et al., 1993; Thornberry et al., 1992). Though overexpression of ICE in many experimental systems results in increased apoptosis (Miura et al., 1993), the results of ICE knockout experiments
rule out the possibility of the involvement of ICE as an indispensable candidate in apoptosis (Kuida et al., 1995). Caspase-1^{−/−} mice are developmentally normal, however caspase-1^{−/−} cells have been reported to be more resistant to apoptosis induced by CD95 ligation (Kuida et al., 1995).

(ii) Caspase-2

Nedd2 was originally identified as a developmentally downregulated gene in mouse brain (Kumar et al, 1994). Caspase-2 deficient mice are developmentally normal, and their cells undergo a normal apoptotic process in response to various stimuli, including nerve growth factor withdrawal (Bergeron et al., 1998). The human homologue, renamed Ich-1 (ICE and ced-3 homologue-1), was subsequently isolated from a human fetal brain cDNA library and shown to encode a protein with sequence homology to ICE and ced-3 (Wang et al., 1994). The inhibitor profile and the substrate specificity of the Nedd2/ Ich-1 gene product have not been explored systematically.

(iii) Caspase-3

Caspase-3 is one of the key executioners of apoptosis being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear repair enzyme PARP (Lazebnik et al., 1994). Caspase-3 is present in cells as a 32 kDa pro-enzyme that is cleaved to 17 kDa active form upon apoptosis (Nicholson et al., 1995). Caspase-3 prefers a DXXD-like substrate whereas caspase-1 prefers a YVAD-like substrate (Nicholson et al., 1995). Caspase-3 knockout mice can survive to birth but they exhibit perinatal mortality as a result of defects in brain development that correlate with a decrease in levels of apoptosis (Kuida et al., 1996).
(iv) **Caspase-8**

Caspase-8 is an upstream caspase involved in the triggering of death cascade in the Fas and TNF-α mediated apoptosis. It has an N-terminal domain with marked identity with the death effector domain (DED) of the adaptor molecule, FADD/MORT1 (Fas-associating protein with death domain) which interacts with CD95 and TNF receptor (Boldin et al., 1996; Muzio et al., 1996). Overexpression of caspase-8 results in apoptosis, and mutation of its catalytic cysteine residue abolishes its apoptotic potential. Caspase-8 knockout mice develop normally until embryonic day 11.5 and then begin to die. Histological examination reveals poorly developed heart musculature (Varfolomeev et al., 1998).

(v) **Caspase-9 (ICE-LAP6 / mch6)**

Caspase-9 is a member of the CED-3 subfamily, bearing high similarity to caspase-3. The major difference between caspase-9 and other family members is the active site pentapeptide QACGG, in which a Glycine is found instead of the usual Arginine. Overexpression of caspase-9, but not of a mutant in which the catalytic cysteine was replaced with an Alanine, induced apoptosis in MCF7 cells.

Caspase-9 knockout mice show abnormalities in brain development similar to those of caspase-3 knockout animals (Kuida et al., 1998; Hakem et al., 1998).

1.4.5 **Caspase-independent cell death**

Caspases are required for the complete manifestations of apoptotic morphology, yet are dispensable for cell death to occur in many systems. Overexpression of Bax or Bak induces apoptosis in the presence of broad-spectrum caspase inhibitors (Xiang et al., 1996;
Mc Carthy et al., 1997). These can also induce mitochondrial dysfunction and kill yeast, which lack endogenous caspases (Zha et al., 1996). AIF, undergoes mitochondrio-nuclear translocation which is caspase independent, resulting in apoptosis (Susin et al., 1996).

1.5 Mitochondria - the central regulatory point in apoptosis

1.5.1 Evidence for the involvement of mitochondria in apoptosis

Mitochondria were regarded as passive organelles that support life, but play no role in apoptosis. The absence of changes in mitochondrial ultrastructure as well as the fact that cells lacking mitochondrial DNA can undergo apoptosis (Jacobson et al., 1993) initially supported the idea that mitochondria do not participate in the process of apoptosis. Recently there had been a paradigm shift in the area of apoptotic research, and the key role, this organelle plays during cell death is now getting unraveled.

The first evidence emphasizing the role of mitochondria in apoptosis came from the studies by Newmeyer et al, who demonstrated using a cell-free system, the requirement of mitochondria-enriched heavy membrane fractions, in Xenopus egg extracts, for the apoptosis to occur (Newmeyer et al., 1994). The fact that members of the Bcl-2 family of proteins regulate cell death, by altering mitochondrial homeostasis (reviewed by Vander Heiden and Thompson, 1999) also suggested a key role for mitochondria in apoptosis.

1.5.2 Release of death promoting factors from mitochondria during apoptosis

Cell death is characterized by the release of various proteins that are normally sequestered in the intermembrane space of mitochondria into the cytosol. Depending on the stimulation, this event can lead to necrosis through irreversible mitochondrial damage and energetic catastrophe or to apoptosis through caspase activation.
(i) **Release of Cytochrome c**

Cytochrome c is encoded in the nucleus and translated on cytoplasmic ribosomes as apo-cytochrome c and follows a unique pathway into mitochondria that does not require the signal sequence, electrochemical potential and general protein translocation machinery (Mayer et al., 1995). The apoprotein in the intermembrane space combines with heme to become the mature protein. Cytochrome c functions as an electron carrier in oxidative phosphorylation, shuffling electrons from complex III to complex IV.

Wang and colleagues demonstrated that addition of dATP to the cell-free extracts from HeLa cells resulted in typical apoptotic changes including activation of caspase-3 and nuclear DNA fragmentation (Liu et al., 1996). Fractionation of the extracts revealed the existence of two factors necessary for the above effect. One of the required factors surprisingly proved to be cytochrome c (Liu et al., 1996). Subsequent investigations demonstrated that following exposure of cells to apoptotic stimuli, cytochrome c is rapidly released from mitochondria to cytosol (Kluck et al., 1997; Yang et al., 1997).

The other factor fractionated from the extracts was the protein Apaf-1 (apoptosis-activating factor 1), a CED-4 homolog in *C. elegans* (Zou et al., 1997). Apaf-1 has an amino terminal caspase-recruitment domain (CARD) and also possesses nucleotide binding site. It was shown that addition of Apaf-1, cytochrome c (Apaf-2) and dATP to pro-caspase-9 (Apaf-3) results in conversion of pro-caspase-9 to active caspase-9. Caspase-9 cause cleavage of pro-caspase-3 to caspase-3, the key enzyme involved in apoptosis (Li et al., 1997).

(ii) **Release of apoptosis inducing factor (AIF)**

Mitochondria isolated from apoptotic cells were demonstrated to induce apoptosis in the acellular system. These results emphasized that mitochondria can effectively control
nuclear apoptosis (Zamzami et al., 1996b). The mitochondria that undergo permeability transition (PT) would liberate a protein (AIF) capable of inducing nuclear apoptosis (Susin et al., 1996). AIF is normally confined to mitochondria, yet subject to mitochondrio-nuclear translocation upon induction of apoptosis by diverse agents (Susin et al., 1999). This nuclear localization of AIF is compatible with the presence of several putative and nuclear localization signals within AIF (Susin et al., 1999). The mitochondrio-nuclear translocation of AIF is caspase-independent. When added to purified nuclei from HeLa cells, recombinant AIF protein induces DNA loss, peripheral chromatin condensation and digestion of chromatin into ~50 kbp fragments but no oligonucleosomal fragmentation is observed (Susin et al., 1999).

(iii) Release of SMAC/DIABLO

Recent investigations identified a mammalian IAP inhibitor, known as Smac (second mitochondria-derived activator of caspases) (Du et al., 2000) or DIABLO (direct IAP-binding protein with low pl) (Verhagen et al., 2000). Smac/DIABLO is a mitochondrial protein, but is released into the cytosol in cells induced to die, presumably following the same exit route as cytochrome c. It binds to IAP family members and neutralizes their anti-apoptotic activity.

1.5.3 Mechanism of cytochrome c release

The exact mechanism resulting in the release of cytochrome c to cytosol is still controversial, but it is evident that Bcl-2 family is intimately involved in the regulation of this process. Two competing models have been proposed to explain how cytochrome c is released from mitochondria during apoptosis.
(i) The first model postulates the opening of a megachannel called the permeability
transition pore (PTP); which is a large conductance, cyclosporin-inhibited channel. This
channel is poorly characterized but is proposed to span both the inner and outer mitochondrial
membranes at sites at which the two membranes are apposed. The adenine nucleotide
translocator (ANT), located in the inner membrane, the voltage-dependent anion channel
(VDAC) found in the outer membrane and the matrix localized cyclophilin D are considered
to be major components of the PTP (reviewed by Loeffler and Kroemer, 2000). Bax was also
found to be a part of this complex (Marzo et al., 1998a). According to this model, PTP
openers, including Bax cause permeabilization of the inner membrane and mitochondrial
depolarization by binding to the ANT (Marzo et al., 1998b). This process allows entry of
water and solutes into the matrix, leading to the swelling and rupture of the outer
mitochondrial membrane allowing cytochrome c and other proteins to be released passively
(permeability transition) (reviewed by Petit et al., 1996).

However, several observations argue against the involvement of PTP in the release of
cytochrome c. It was shown that cyclosporin A does not block Bax- or Bid-induced
cytochrome c release from mitochondria (Eskes et al., 1998). Cytochrome c has also been
reported to occur before a drop in mitochondrial membrane potential which reflects PTP
opening (Bossy-Wetzel et al., 1998) and during apoptosis of several cell types, mitochondria
do not swell but rather shrink, a process described as "mitochondrial pyknosis" (Mancini et
al., 1997).

(ii) The second model envisages the formation of a cytochrome c conducting channel in
the outer mitochondrial membrane regulated by Bcl-2 family of proteins. The clue that Bcl-2
members form channels came from the three dimensional structure of Bcl-X\textsubscript{L} and Bid, which
resembles the structure of pore-forming domains of diptheria toxin and some bacterial
colicins (Muchmore et al., 1996). A likely candidate for the formation of this channel is the pro-apoptotic protein, Bax. It was shown that an oligomer of Bax can form large-conductance channels in lipid bilayers (Schlessinger et al., 1997; Antonsson et al., 1997). Moreover, addition of Bax directly to isolated mitochondria triggers release of cytochrome c through a mechanism insensitive to PT blockers (Eskes et al., 1998).

Yet another model involves Bax cooperating with VDAC to form a cytochrome c-conducting channel (Henchoz et al., 1997). Following binding of Bax to the VDAC, the conformation of the VDAC would change, leading to the formation of a channel that is permeable to cytochrome c. But there is no evidence, as of now that Bcl-2 family proteins form channels in vivo.

1.5.4 Regulation of mitochondrial homeostasis and cell death by Bcl-2 family of proteins

The Bcl-2 family of proteins constitute one of the most crucial classes of apoptosis regulatory proteins. These include both the pro-apoptotic (Bax, Bad, Bak, Bid, Bcl-X\textsubscript{s}, Bik, Bim) and anti-apoptotic (Bcl-2, Bcl-X\textsubscript{L}, Bcl\textsubscript{w}, Mcl\textsubscript{1}) proteins (Gross et al., 1999; reviewed by Kroemer, 1997). These proteins are mostly localized to the outer membranes of mitochondria, endoplasmic reticulum and nucleus, as a result of a carboxy terminal membrane anchor (reviewed by Yang and Korsmeyer, 1996). Some of them are cytosolic (Bax, Bcl-X\textsubscript{L}, Bid, Bad and Bim) but translocate to mitochondria during apoptosis (Hsu et al., 1997; Desagher et al., 1999; Puthalakath et al., 1999; reviewed by Downward 1999). Members of this family are capable of dimerization and the ratio of pro- to anti-apoptotic molecules determine, in part, the susceptibility of cells to undergo programmed cell death (Oltvai et al., 1993). These proteins regulate apoptosis in part by affecting the mitochondrial
compartmentalization of cytochrome c. Expression of Bcl-2 or Bcl-XL prevents the redistribution of cytochrome c in response to multiple death inducing stimuli (Yang et al., 1997; Kluck et al., 1997; Vander Heiden et al., 1997), while Bax promotes cytochrome c release (Manon et al., 1997; Rosse et al., 1997). Bcl-2 expression inhibits the generation of reactive oxygen species (Hockenbery et al., 1993; Kane et al., 1993) and intracellular acidification (Gottlieb et al., 1996) and stabilizes the mitochondrial membrane potential (Vander Heiden et al., 1997; Zamzami et al., 1995a). Bcl-2 can also effect mitochondrial proton flux (Shimizu et al., 1998) and modulate calcium homeostasis (Zhu et al., 1999).

1.5.5 Mitochondria-dependent and -independent pathways

In some type of cells, apoptosis triggered by Fas (CD95) and certain members of the TNF family of death receptors is not blocked by overexpression of Bcl-2 or other anti-apoptotic members of the Bcl-2 family, circumventing the participation of mitochondria or other organelles, where Bcl-2 and many of its homologues reside as integral membrane proteins (reviewed by Vaux and Strasser, 1996) (type I cells).

However in some cells, Bcl-2 is capable of suppressing Fas- or TNF-induced apoptosis (type II cells). The basis for this difference in Bcl-2 sensitivity appears to reside in whether caspase-8 does or does not require a mitochondria-dependent amplification step to achieve sufficient activation of downstream effector caspases for apoptosis. Addition of caspase-8 at high concentration to cytosolic extracts results in downstream caspase activation and apoptosis, whereas if lower concentrations of caspase-8 are added, the downstream caspase activation is minimal, unless mitochondria are added to cytosolic extracts (Kuwana et al., 1998).
The mitochondrial-dependent and -independent pathways in mammalian cells
1.6 The Fas and TNF-α signal transduction - a prototype of cell death pathway

Fas (CD95) and TNF receptor 1 (TNFR1) are cell surface receptors that when trimerized with ligand (TNF-α and FasL, respectively), induce apoptosis (reviewed by Cleveland and Ihle, 1995; Nagata and Goldstein, 1995). These pathways play a critical role in regulation of the immune system. Inappropriate expression of the FasL can enable tumor cells to escape immune surveillance (Hahne et al., 1996) and expression is usually restricted to sites of immune privilege (Griffith et al., 1995).

Apoptotic signaling by Fas and TNFR1 is mediated by a stretch of 80 amino acids, called the death domain (DD) in the cytoplasmic portion of each receptor. Adaptor proteins (FADD, Mort 1, RIP and TRADD) bind to these DDs via their own DDs (Boldin et al., 1995; Chinnaiyan et al., 1995; Chinnaiyan et al., 1996b; Stanger et al., 1995). Overexpression of FADD and RIP causes apoptosis. FADD also contains a motif at its amino terminus, the death effector domain (DED) which binds to the pro-domain of caspase-8. A complex of proteins are found associated with stimulated CD95 (Designated as CAP 1-4) (Kischkel et al., 1995). Together with CD95 and caspase-8, these proteins form a complex called the death-inducing signaling complex (DISC). CAP4 contain two DEDs at its N-terminus and showed the typical domain structure of an ICE-like protease at its C-terminus. It was therefore termed FLICE (for FADD-like ICE), now called caspase-8 (Alnemri et al., 1996). After stimulation FADD, Mort 1 and caspase-8 are recruited to CD95 within seconds after receptor engagement. Direct binding of caspase-8 causes structural changes in the molecule that result in the autoproteolytic activation. The active subunits p10 and p18 are released into the cytoplasm. The active caspase-8 cleaves various cellular death substrates, including other caspases, such as caspase-3, thus initiating the execution of apoptosis.
There are other DED containing proteins, some virally encoded, called the v-FLIPS (for viral FLICE inhibitory proteins). vFLIPS consist of two DEDs and biochemical analysis of v-FLIP-transfected cells showed that they bind to the CD95/FADD complex and thus inhibit the recruitment of caspase-8 and a functional DISC formation (Thome et al, 1997).

1.7 The TRAIL (APO-2L) apoptosis system

TRAIL or TNF-related apoptosis-inducing ligand was identified on the basis of sequence homology to the other members of the TNF family (Wiley et al, 1995). TRAIL was found to induce apoptosis more efficiently in tumor cells than in normal cells. TRAIL can bind two apoptosis-inducing receptors, TRAIL-R1 (DR4) and TRAIL-R2 (Killer, DR5, TRICK2), two additional cell-bound receptors, incapable of transmitting an apoptotic signal, TRAIL-R3 and TRAIL-R4 (reviewed by Degli-Esposti, 1999). It was earlier thought that the existence of these functionally distinct receptors might provide an answer to the differential sensitivity to TRAIL, observed between normal and transformed cells, which was later disproved (reviewed by Walczak and Krammer, 2000).

The physiological significance of this novel apoptosis-inducing system remained unknown for a long time. Interestingly, functional surface expression of TRAIL was often associated with stimulation by interferons. Therefore, it is likely that the antitumoral effect of IFNs may at least be partially mediated by TRAIL-induced direct killing of TRAIL-sensitive tumor cells (Sedger et al, 1999). A number of studies have been performed combining TRAIL with different chemotherapeutic drugs for the treatment of various malignancies (reviewed by Bonavida et al, 1999). Most chemotherapeutic drugs and radiation therapy used in the treatment of malignancies lead to apoptosis primarily by engagement of the mitochondrial proapoptotic machinery. TRAIL induces apoptosis via a caspase signaling
cascade that executes apoptosis independently of the proapoptotic machinery of mitochondria. TRAIL can bypass the anti-apoptotic effect of Bcl-2 or Bcl-XL overexpression. TRAIL and chemotherapeutic drugs work via distinct apoptotic pathways and such a combinatorial treatment of cancer will most likely diminish the chances of the tumor to develop further.

1.8 Apoptosis and p53

The tumor suppressor protein p53 plays multiple roles in cells. Expression of high levels of wild type (but not mutant) p53 has two outcomes: cell cycle arrest or apoptosis. Both provide mechanisms by which p53 functions to control DNA damage, protecting cellular descendants from accumulating excessive mutations. In response to irradiation or other DNA-damaging insults, p53 levels rise and produce G1 arrest (Kastan et al., 1992). Possible mechanisms include p53-mediated transcription of genes such as p21 / Waf / Cip, which inhibit cyclin / cdk activity and thereby inhibit cyclin-dependent entry into S phase, as well as DNA replication machinery (reviewed by Pines, 1994).

p53 mediates apoptosis in several cell types induced by different stimuli such as DNA damage (Clarke et al., 1993) adenovirus E1A expression (Debbas and White, 1993), myc expression (Hermeking and Eick, 1994) or withdrawal of growth factors (Johnson et al., 1993). However apoptosis can also occur by p53-independent pathways (Clarke et al., 1993; Lowe et al., 1993). The p53-dependent apoptotic pathway has been shown to be an important mechanism by which transformation is suppressed in oncogene-expressing cells (Lowe et al., 1994).
1.9 Programmed cell death in invertebrates

(i) PCD in C. elegans

The existence of an intrinsic cell suicide program was ascertained through genetic studies in the nematode *C. elegans* that identified genes involved in the cell death program and its control (reviewed by Ellis and Horvitz, 1986; Horvitz and Ellis, 1982). The genes CED-3 and CED-4 (the homologues of ICE and Apaf-1 in mammals) are required for execution of cell death and CED-9 (homologue of Bcl-2 in mammals) inhibits cell death in *C. elegans* (Hengartner and Horvitz, 1992; Yuan and Horvitz 1992; Yuan et al., 1993). Mutational analysis of these genes in *C. elegans* have defined a sequential death pathway. Using yeast-two hybrid analysis it was demonstrated that CED-4 can bind to CED-9, CED-3 or both simultaneously (Chinnaiyan et al., 1997; Spector et al., 1997) analogous to the mammalian 'apoptosome' consisting of Apaf-1, Bcl-XL and caspase-9 (Pan et al., 1998), highlighting the functional similarity of the worm and human cell death machinery.

(ii) PCD in Drosophila

Many death regulatory genes have been identified in *Drosophila* through mutation analysis. The first global cell death-defective *Drosophila* mutation was discovered by following apoptosis in living fly embryos (White et al., 1994). This rare phenotype was mapped to a complex locus, the Reaper region. Later three cell-death activators were discovered *Reaper* (*Rpr*), *Grim* and *Hid* which mapped to the same locus (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Cell death induced by each of these proteins are preceded by caspase activation (Chen et al., 1998) and inhibited by caspase inhibitors. Caspases characterized in flies include Dredd (Chen et al., 1998) and Dronc (Dorstyn et al., 1999) which resemble effector caspases and DCP-1 (Song et al., 1997) and Drice (Fraser and
Evan, 1997) resembling executioner caspases. Dark - a fly homologue of Apaf-1/CED-4 relays death signals to apoptotic caspases (Rodriguez et al., 1999).

(iii) **PCD in plants, Fungi and bacteria**

The cell death associated with the hypersensitive response (HR) in several plant-pathogen interactions has morphological similarities to mammalian apoptosis (reviewed by Greenberg, 1996). Caspase-like proteolytic activity was detected in tobacco plant tissues that were developing HR following infection with tobacco mosaic virus (TMV) (reviewed by Mittler and Lam, 1996). In addition, some types of plant cell death are accompanied by DNA damage often with the characteristics of endonucleotically processed DNA, one of the hallmarks of apoptosis (Wyllie, 1980; reviewed by Kerr et al., 1972).

The pro-apoptotic proteins Bax and Bak are capable of provoking death of the yeast *Saccharomyces pombe* (Jürgensmeier et al., 1997). The expression of Bax in the yeast *S. cerevisiae* is lethal in the presence of a functional respiratory chain and Bcl-2 inhibits the yeast cell death (Greenhalf et al., 1996). Furthermore, mitochondria of Bax-expressing yeast cells release cytochrome c which is inhibited by coexpression of Bcl-XL (Manon et al., 1997). These observations suggest that some elements involved in mammalian cell death exist in yeasts.

The best characterized programmed cell death mechanism of bacteria are found under starvation conditions where moribund cells are programmed to release their contents by lysis, thereby providing nutrients to the remaining healthy cells in the population, termed GASP (growth advantage in stationary phase). The 'entericidin locus' of *E. coli* governs stationary phase bacteriolysis (Bishop et al., 1998).
1.10 De-regulation of apoptosis and disease

Diseases characterized by accumulation of cells include cancer, autoimmune diseases 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 (reviewed by Thomspn, 1995).

Excessive cell death can result from acquired or genetic conditions that enhance the accumulation of signals that induce apoptosis or that decrease the threshold at which such events induce apoptosis. These include virus-induced lymphocyte depletion in AIDS, cell death in neuro-degenerative disorders like Alzheimer's disease, Parkinson's disease, retinitis pigmentosa and various forms of cerebral degeneration (reviewed by Thompson, 1995).