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# Apoptosis in health, disease, and therapy: overview and methodology

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## 1.1 Introduction: life cannot exist without cellular death

Apoptosis, or programmed cell death, is the mechanism by which most cells die both physiologically and pathologically. The realization in the mid 1980s that cells die by an active, genetically defined process changed not only our views on cellular life but led to a whole new discipline of biologic study with significant implications for medicine (Thompson, 1995; Robertson *et al.*, 2002). Apoptosis research has advanced our understanding of a basic cellular process, shed insight into many diseases, and is poised to affect the future practice of medicine by the introduction of therapies targeting this cell death process.

In this book, the term "apoptosis" is used synonymously, for right or wrong, with programmed cell death (PCD). While PCD may be a more appropriate term, encompassing all forms of active physiological cell death, apoptosis, which is defined morphologically and biochemically, is used here for historical purposes (Lockshin and Zakeri, 2002; Melino, 2002; Sloviter, 2002). The original "anatomical" characteristics of apoptosis were noted in the nineteenth century (reviewed in Clarke and Clarke, 1996; Rich *et al.*, 1999). However, it was not until publications in 1951 and in the 1960s described developmental cell death or "shrinkage necrosis" that the PCD concept was recognized, reintroduced, and formalized (Lockshin and Zakeri, 2001; Kerr, 2002; Vaux, 2002). The term "apoptosis" was coined in 1972, referring to this morphologically defined form of cell death (Kerr *et al.*, 1972). However, a broader recognition of the

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centrality and import of PCD did not occur until seminal studies performed by Horvitz and colleagues in the early 1980s demonstrated the genetic underpinnings of this process in the nematode, *Caenorhabditis elegans* (see Chapter 2; Ellis and Horvitz, 1986). The impact of these genetic discoveries was recently recognized by the awarding of the 2002 Nobel Prize in Physiology or Medicine to Sidney Brenner, John E. Sulston, and H. Robert Horvitz (Benitez-Bribiesca, 2003).

### 1.1.1 Apoptosis characteristics

Apoptosis is the physiological mechanism by which cells die, characterized by the features listed in Table 1.1, and is distinct from the accidental form of cell death called necrosis which represents an "extreme" in the continuum of cell death. Although initial work sought to characterize cell death as strictly apoptotic or necrotic, many cell death events display both apoptotic and necrotic features. Thus, necrosis represents one extreme of a continuum, with classical apoptosis at the other. In certain cell types and tissues, such as neurons, cell death usually proceeds with unique characteristics that generated long debate with respect to mechanism. Many researchers now recognize that all features of apoptosis need not be present. In necrosis, there is a loss of cellular plasma membrane integrity accompanied by an inflammatory response, while in apoptosis the plasma membrane integrity is preserved and the cells are discretely disassembled and phagocytosed without an inflammatory response. Necrosis occurs in severe circumstances, such as cases of sudden transfer of energy (kinetic, electrical, thermal), frostbite, or exposure to certain toxins. These can rupture the plasma membrane and/or severely compromise the cells' energy-producing respiratory process. However, the normal physiological route to cell death is, under most circumstances, apoptosis. Only under extreme conditions, when the cell cannot properly execute its apoptotic program, does the cell die, by default, by necrosis, thus blurring the distinction between these two forms of cell death. While necrosis can be considered pathophysiological, it is a medically undesired form of cell death because the ensuing inflammatory response causes secondary cell death and tissue damage. For example, the acute use of high-dose steroid therapy to suppress inflammation in spinal cord injury has led to improvements in neurologic outcomes by suppressing some of this secondary cell death. Arguments for a physiologic, and potentially beneficial, role of necrosis have also been made (Proskuryakov *et al.*, 2003).

### 1.1.2 Apoptosis pathways

There exist multiple cellular pathways triggering apoptosis, two of which, the extrinsic and intrinsic pathways, are the best studied. Various other pathways exist, and

**Table 1.1** *Characteristics distinguishing apoptosis from necrosis*

| Defining features | Apoptosis | Necrosis |
|---|---|---|
| *Physiologic/pathologic features* | | |
| Cellular role | Usually normal | Abnormal, accidental |
| Process | Active, energy dependent | Passive, results from lack/loss of energy |
| Distribution | Dispersed, affects individual cells | Contiguous, simultaneous, and massive affects in damaged tissue areas |
| Triggers | 100s of physiologic and noxious stimuli | Sudden transfer of energy, specific toxins, or ATP depletion |
| Induction | Slow (hours), stochastic | Rapid (seconds, minutes) |
| Tissue inflammation | Absent | Present |
| Cell removal | Rapid and discrete | Slow |
| *Morphologic features* | | |
| Cellular membranes | Integrity preserved, blebbing of intact plasma membrane | Loss of integrity, with spilling of cell constituents |
| Cell volume | Decreased, as well as the formation of small, fragmented "apoptotic bodies" or inclusions | Increased |
| Organelle structure | Late preservation, with exception of nuclear condensation and fragmentation | Swelling of nucleus and other organelles |
| Chromatin | Discrete, organized condensation, margination and fragmentation (e.g. pyknotic nuclei) | Pattern conserved |
| *Biochemical and molecular features* | | |
| Mitochondrial permeability transition | Moderate | Severe |
| Mitochondrial membrane potential (delta psi-m) | Transient loss | Permanent loss |
| Requirement for ATP | Yes | No |
| Membrane phospholipid asymmetry | Exteriorization of phosphatidylserine from inner to outer leaflet of plasma membrane | Unchanged |
| Cell pH | Acidification | Unchanged |
| DNA cleavage | Initial specific large cleavage products of 300, then 50, kbp, followed by internucleosomal cleavage leading to DNA ladder pattern of 180 bp unit repeats | Random DNA cleavage |
| Caspase dependence | Yes | No |

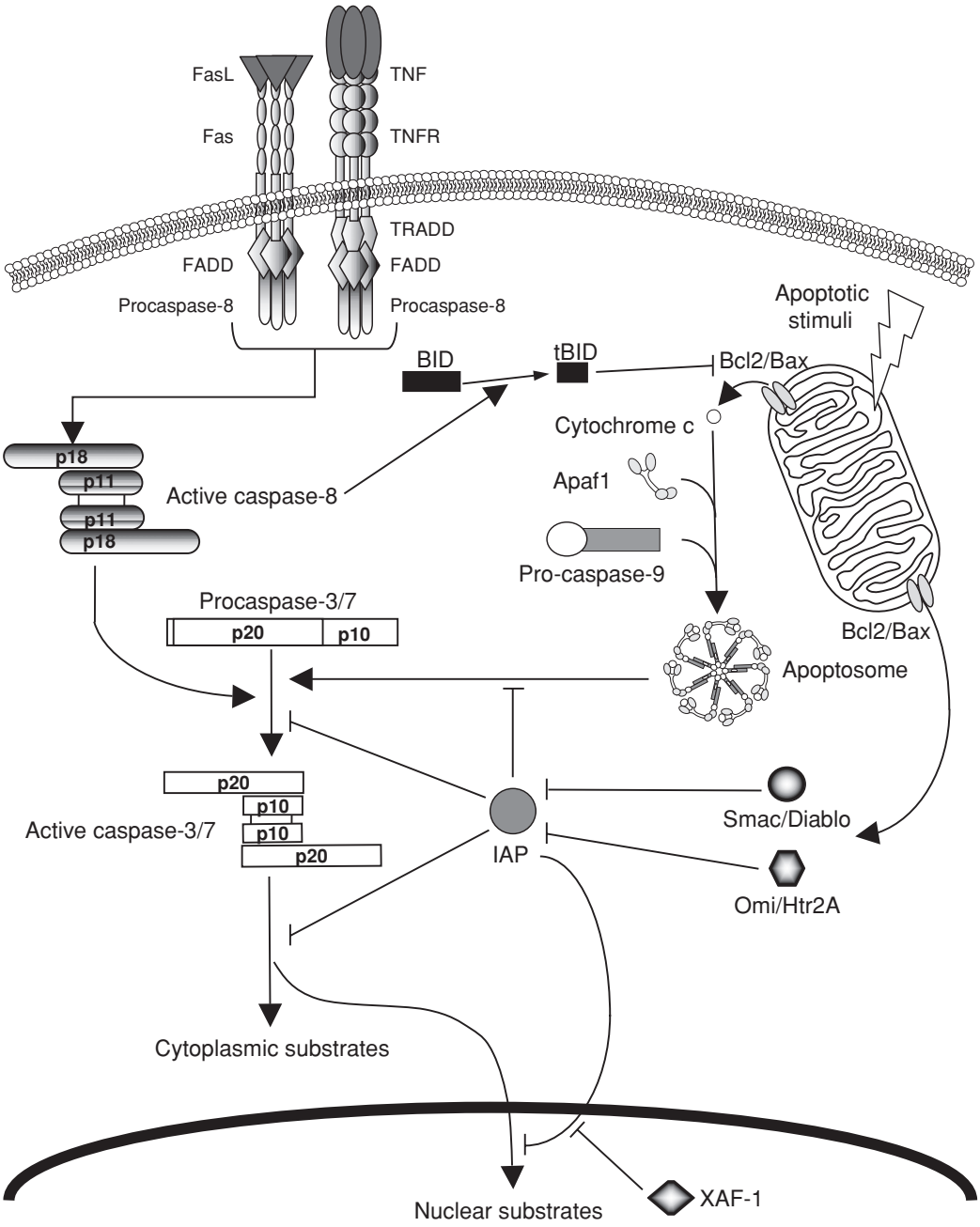


Figure 1.1 *Intrinsic and extrinsic apoptotic pathways in mammalian cells.* Intracellular stress results in the activation of the mitochondrial, or intrinsic, pathway which leads to cytochrome c release, apoptosome formation, and caspase activation. Extracellular ligand binding to death receptors triggers the extrinsic pathways that can either directly result in the activation of the caspases, or requires further amplification through the mitochondrial pathway dependent

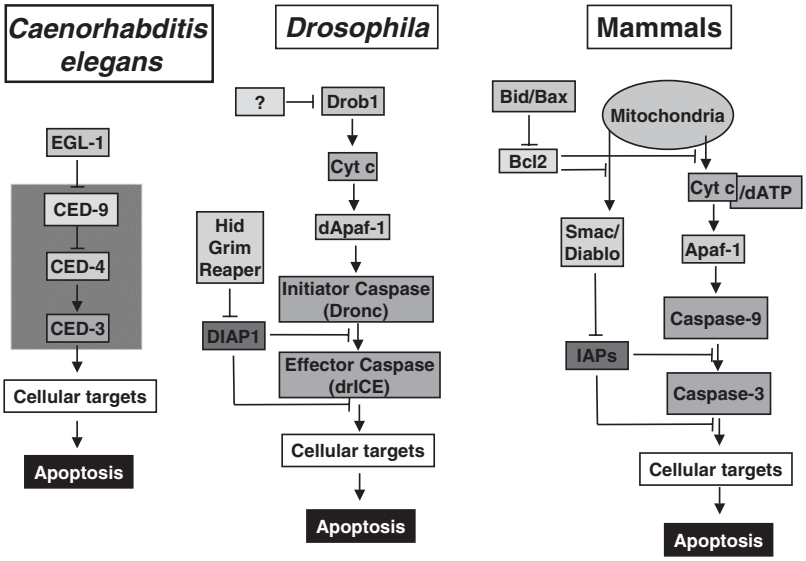


Figure 1.2 *Core elements of the apoptotic pathways conserved across several phyla*. The core apoptotic machinery is shown for *C. elegans, D. melanogaster*, and for mammals. The original core of death genes identified in *C. elegans* (ced-3, ced-4, and ced-9) are shown boxed in gray (Ellis and Horvitz, 1986; Hengartner *et al*., 1992). The shading scheme for each gene identifies orthologs and paralogs across the phyla. For additional information see chapter 2, as well as references by Meier *et al*., 2000, Aravind *et al*., 2001, and Lawen, 2003.

some are discussed in a review by Ferri and Kroemer (2001). The extrinsic pathway involves plasma membrane receptors of the tumor necrosis factor receptor (TNFR) superfamily that recognize extracellular death-inducing ligands, while the intrinsic pathway utilizes the coordinated control and release of apoptogenic factors from the mitochondria which "sense" many death-inducing stimuli through regulatory factors of the bcl2 family (Figure 1.1). All known death pathways culminate in the activation of a proteolytic cascade involving a family of proteases, the caspases (Figures 1.1 and 1.2). The caspases are cysteinyl-containing active center proteases with specificity for protein cleavage after aspartyl residues (Thornberry and Lazebnik, 1998; Earnshaw *et al*., 1999; Nicholson, 1999). Thus, the term *caspases* for cysteinyl-containing aspartate-specific proteinase. The caspases are responsible

← on the cell type. Both apoptotic signaling pathways converge at the level of effector caspases, such as caspase-3 and -7. Multiple control points exist along these pathways, controlling either the release of cytochrome c and other apoptogenic factors from the mitochondria or by regulating the caspase inhibitors, the inhibitors of apoptosis (IAPs), through their antagonists or through other regulatory mechanisms.

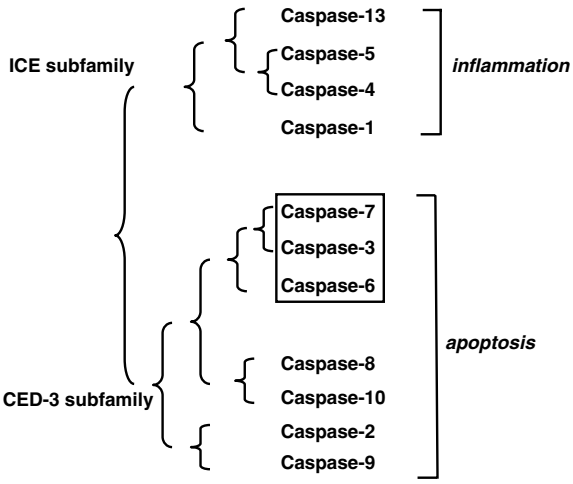


Figure 1.3 *The caspase family*. Phylogenetic analysis segregates the human caspases into two major subfamilies, one based on caspase-1 previously referred to as ICE, for interleukin-converting enzyme, and the other based on similarities to the *Caenorhabditis elegans* cell death gene, *ced-3*. Further classification of the caspases is possible: into those that mediate cytokine maturation that are involved in inflammation, those with a short pro-domain involved in the effector phase of apoptosis (shown boxed), and those with a long pro-domain and involved in the initiator phase of apoptosis (not boxed). Note evolutionary distances are not accurately represented in this dendrogram. Based on Nicholson, 1999.

for many of the hallmarks of apoptosis listed in Table 1.1, through their cleavage of specific polypeptide substrates (Fischer *et al.*, 2003). The caspases – e.g. caspase-2, -3, -6, -7, -8, -9, -10 (and 12 in the mouse) – are not the only proteases involved in PCD; calpains have been shown to have a role in some instances, and there is the possibility of others as yet undiscovered. However, the caspases are the prime effectors of apoptosis, and their activation produces a catalytic cascade leading to further caspase activation, ultimately committing the cell to death by cleavage of structural proteins, activation of nucleases, as well as the resultant inactivation of survival, repair, or anti-apoptotic factors and activation of pro-death factors. Note that not all caspases are involved in apoptosis and that certain caspases are involved in the processing of pro-inflammatory cytokines, e.g. caspase-1, -4, -5, and -11 (Figure 1.3). In addition, certain caspases may play highly specific roles in normal cell differentiation and maturation, such as the enucleation of red blood cells, formation of lens cells of the eye, or platelet formation from megakaryocytes, or in blast phase activation of lymphocytes, apparently without inducing apoptosis (see Chapter 2; Ishizaki *et al.*, 1998; Weil *et al.*, 1998; Alam *et al.*, 1999; Kennedy *et al.*, 1999; Zeuner *et al.*, 1999; De Botton *et al.*, 2002; Newton and Strasser, 2003; Olson *et al.*, 2003; Perfettini and Kroemer, 2003; Philchenkov, 2003).

### 1.1.3 Conservation and diversification of the apoptosis machinery

Caspases exist within the cell as inactive zymogens and are activated primarily by two distinct mechanisms both involving protein–protein interactions within large complexes and proximity-induced processing of the caspases (Chen *et al.*, 2002; Boatright *et al.*, 2003; Chang *et al.*, 2003). One mechanism involves the generation of the DISC, the death-inducing signaling complex, which is formed by the trimerization of plasma membrane death receptors upon binding of death ligands, such as TNFα or TNFα-related apoptosis-inducing ligand (TRAIL). Receptor trimerization subsequently triggers the recruitment of adaptor molecules and initiator or apical caspases, such as caspase-8 or -10, to the cytoplasmic portion of the death receptors. This induced proximity then allows for activation of the apical caspase, which can then in turn activate downstream effector caspases. The second mechanism involves the release of apoptogenic factors from the mitochondria, particularly cytochrome c, which results in the formation of the apoptosome, a large helical complex comprised of apaf-1, cytochrome c, and caspase-9 (Acehan *et al.*, 2002; Shi, 2002). This complex formation induces a conformational shift and allows the proximity-induced activation of the apical caspase-9, which can then cleave downstream effector caspases, such as caspase-3 and -7. The two death pathways do not function in isolation with evidence of both cross-talk and feed-forward loops. Additional, less well-understood pathways and mechanisms for caspase activation are also found (Nakagawa *et al.*, 2000; Sperandio *et al.*, 2000; Ferri and Kroemer, 2001; Forcet *et al.*, 2001; Troy *et al.*, 2001; Rao *et al.*, 2002; Read *et al.*, 2002; Chandra and Tang, 2003). A third characterized pathway of caspase activation that is specific to cytotoxic T cell-mediated cell death is reviewed in Chapter 6. This form of cell death results from the introduction of the serine proteases, known as granzymes, into the cytoplasm which can then either directly activate the caspase cascade (by cleavage of specific caspases or bid) or bypass the caspases (by cleaving their substrates directly) (Barry and Bleackley, 2002; Lieberman, 2003).

The cell is armed with an elaborate self-destruct mechanism, comprised of inactive zymogens that can be activated by numerous stresses or triggers, but which remain tightly controlled (e.g. Fussenegger *et al.*, 2000; Bortner and Cidlowski, 2002). For example, the cell contains endogenous caspase inhibitors, the inhibitors of apoptosis (IAPs) (Miller, 1999; Verhagen *et al.*, 2001; Salvesen and Duckett, 2002; Shi, 2002; Stennicke *et al.*, 2002), as well as Bcl-2 family regulators of the mitochondrial release of apoptogenic factors (Gross *et al.*, 1999; Wang, 2001; Newmeyer and Ferguson-Miller, 2003; Scorrano and Korsmeyer, 2003; Tsujimoto, 2003), and modulators of DISC signaling, such as FLIP (Thome and Tschopp, 2001; Peter and Krammer, 2003). All these factors are controlled by several different mechanisms, including transcriptional and post-transcriptional regulation, translational

control, phosphorylation, proteolysis, and ubiquitin-mediated protein degradation. In addition, the caspase-inhibiting IAPs are antagonized by mitochondrial factors (i.e. SMAC/DIABLO – Du *et al.*, 2000, Verhagen *et al.*, 2000 – and OMI/HTra2 – Suzuki *et al.*, 2001), cytoplasmic proteins (i.e. eRF3 – Hegde *et al.*, 2003), and nuclear factors (i.e. XAF-1 – Liston *et al.*, 2001), while the bcl-2 anti-apoptotic proteins are antagonized by the BH3-only and Bax-like members of the bcl-2 family (Figure 1.1). Many of these protein interactions have been solved structurally (Fesik, 2000; Shi, 2001). Thus, within the cell, there exists a highly regulated and structured machinery to induce or suppress cell death. The identity and interplay of factors controlling this ongoing internal conflict form the basis of an enormous amount of basic, pre-clinical, and clinical research.

Multicellular organisms are remarkable in their complexity. They are characterized by an almost unimaginable diverse array of molecular mechanisms which enable the cellular propagation, differentiation, and maintenance which underpin eukaryotic life forms. But just as there exist wide-ranging, precisely programmed mechanisms to generate the constituent cellular components, so too there exist elaborate means of ending the lives of cells. The eukaryotic milieu is of necessity an intolerant one – cells have a narrow scope of appropriate behavior, deviation from which rapidly results in cellular death. Thus, cells which have served their purpose in development, cells which have reached the end of their natural life span post-development, and cells which have sustained an injury or have in some way become dysregulated die efficiently; they commit suicide and are disassembled. Although the series of mechanisms enacting this cellular attrition pale in comparison with those required to generate a cell, as might be expected, they are nonetheless remarkably complex. As logical as this state of affairs may appear to be in hindsight, a full appreciation has only come over the last 15 years.

Included in the many factors involved in apoptosis control are protein motifs such as bcl2 homology regions 1–4 (BH1, BH2, BH3, and BH4) and baculovirus IAP repeats (BIR domains), as well as CARD, DD, DED, NACHT, and PYRIN domains or motifs (see abbreviations and references in Table 1.2). These motifs or domains are not exclusive to apoptosis; many are found in proteins that have roles in immunity, in particular reflecting the co-evolution of the two processes (see Section 1.2 and Chapters 6 and 7; Bertin and DiStefano, 2000; Koonin and Aravind, 2000; Pawlowski *et al.*, 2001; Staub *et al.*, 2001; Bouchier-Hayes and Martin, 2002; Gumucio *et al.*, 2002; Martinon *et al.*, 2002; Chamaillard *et al.*, 2003; Creagh *et al.*, 2003). The number of genes known to contain these motifs has grown with the completion of the human genome project, with more than 100 genes identified to date (Table 1.2). Among the many other factors involved in apoptosis are those that appear to exist as "orphans" (cytochrome c) or as small family clusters (p53, p63, and p73), only one member of which may play a predominant role in apoptosis control.

**Table 1.2** *Death gene motifs and numbers of human genes*

| Gene family based on homology or motif [InterPro accession number] | Number of genes identified* (at 06/2003) | References (some key references listed at bottom) |
|---|---|---|
| **Caspase** [IPR001309, IPR002138] | 11 | Lamkanfi *et al.*, 2002 |
| **Bcl-2** (BH domains, includes BH3-only members) [IPR000712, IPR003093, IPR002475] | 25 | |
| **IAP** (BIR-containing) [IPR001370] | 8 | Salvesen and Duckett, 2002 |
| **TNFR** (TNF receptor family) [IPR001368] | 29 | Aggarwal, 2003 |
| **TNF ligands** [IPR001875] | 18 | Aggarwal, 2003 |
| **DED** (death effector domain) [IPR001875] | 12 | Tibbetts *et al.*, 2003 |
| **DD** (death domain) [IPR000488] | 33 | |
| **CARD** (caspase recruitment domain) [IPR001315] | 25 | Hofmann *et al.*, 1997; Bouchier-Hayes and Martin, 2002 |
| **NACHT** [IPR007111] | 20 | Koonin and Aravind, 2000 |
| **NOD** (nucleotide-binding oligomerization domain) | 24 | Inohara and Nunez, 2003 |
| **NALPS** (a "caterpiller" sub-domain; includes PYPAF) | 14 | Tschopp *et al.*, 2003; Harton *et al.*, 2002 |
| **PAAD** (includes PYRIN or PYD, and DAPIN) [IPR004020] | 19 | Bertin and DiStefano, 2000; Fairbrother *et al.*, 2001; Pawlowski *et al.*, 2001; Staub *et al.*, 2001 |

IAP, inhibitor-of-apoptosis; BH, bcl-2 homology domain; BIR, baculovirus IAP repeat; CATERPILLER, CARD/Transcription enhancer/R(purine)-binding/pyrin/lots of leucine repeats; NACHT, NAIP/CIITA/HET-E/TP1; NOD, nucleotide-binding oligomerization domain; PAAD, Pyrin/AIM/ASC/DD-like; PYD, pyrin domain; PYPAF, pyrin-containing APAF1-like; TNF, tumor necrosis factor. *These categories are based on protein families, or specific motifs, and are not mutually exclusive, with some proteins possessing two or more different motifs. Not all the proteins containing these motifs are involved in apoptosis; many are involved in immunity and inflammation and other functions as well. The number of genes is a conservative estimate based on published reports. The true number of genes is likely to be different, and for the most part higher than those stated, based on some bioinformatic analysis (see below for examples and caveats) and due to conservative estimates of the predicted total number of genes. The EnsMART/EnsemblMART data mining tool (Clamp *et al.*, 2003) at Ensembl (www.ensemble.org) was used to create a non-redundant list of human gene products that contained specific domains as described by InterPro (www.ebi.ac.uk/interpro/), a database for protein families, domains, and functional sites (Mulder *et al.*, 2003). Lists of genes, sequences, and descriptions can be output from any of the nine species in EnsMART with a variety of filter options from the predicted genes of the complete genomes in an easy three-step process on the Ensembl website. For example, an analysis of the human genome for the death domain (*DD* motif) identified 31 genes compared with the 33 listed here, while the baculovirus *IAP* repeat (BIR motif) identified 11 genes compared with the eight listed here. The three additional IAP genes are hILP3, a likely XIAP expressed pseudogene, and two NAIP-like sequences which also represent expressed pseudogenes (Xu *et al.*, 2002). Key reviews and references include: Koonin and Aravind, 2002; Reed, 2002; Doctor *et al.*, 2003; Liu *et al.*, 2003; Reed *et al.*, 2003.

Genetic ablation of genes involved in apoptosis, mainly in mice, has demonstrated the necessity of cell death in the shaping of metazoan life, such as the genes involved in blastocyst cavitation (Joza *et al.*, 2001).

In hindsight, it seems logical that the death of a cell should be as important as its birth, thus completing the circle of life. In fact, proliferation and apoptosis are intimately linked, as errors during cell cycle progression will result in a default pathway to apoptosis. Questions still remain as to when particular apoptotic processes evolved and if unicellular organisms die by some form of PCD (Ameisen, 2002; Koonin and Aravind, 2002; Huettenbrenner *et al.*, 2003). Although some of the death motif-containing genes exist in yeast, they are likely to be involved in functions other then cell death (e.g. BIR domain-containing proteins that play a role in cytokinesis rather than apoptosis control; Miller, 1999; Silke and Vaux, 2001). While caspases are a central part of apoptosis in metazoans, no counterpart has yet to be identified in plants, but metacaspases or other proteases may fulfill this role (Ameisen, 2002; Koonin and Aravind, 2002; Hoeberichts and Woltering, 2003). Nevertheless, it is becoming clear that even plant cells can undergo apoptosis and that many of the genes involved in apoptosis control in mammals also work in transgenic plants, with dramatic results (Dickman *et al.*, 2001).

The sequencing of several metazoan genomes has brought a greater appreciation of the true complexity involved in the control of apoptosis, with many newly identified genes sub-serving more specialized and frequently redundant roles, increasing in parallel with the complexity of the organism (Table 1.3 and Chapter 2). This redundancy has confounded the analysis of many murine strains with ablated apoptotic loci, with compensatory changes often attenuating the predicted outcome (e.g. Holcik *et al.*, 2000; Zheng *et al.*, 2000; Harlin *et al.*, 2001).

The importance of several of these apoptosis gene families is demonstrated by the subversion of these pathways by viruses (see Chapter 7). Viruses use various strategies, most likely appropriated from the host cell, to suppress (or induce in some cases) the normal cellular response to viral infection, such as the induction of host cell apoptosis or the shut down of protein synthesis. Many examples of viral proteins targeting p53, the mitochondria (e.g. bcl-2 homologs), the TNFR family, caspases, NFkB (nuclear factor κB), IFN (interferon), and protein kinase R (PKR) exist, illustrating the key role these nodes play in the "apoptosis" pathway. Viruses have taught us much about the fundamental aspects of cellular physiology.

## 1.2 Apoptosis: roles in health

Apoptosis is a normal physiologic process beginning with the deletion, fusion, and sculpting of structures during embryogenesis such as in the removal of cells