1 Why be interested in death?

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1. Introduction

The concept that a dead cell can provide answers to several critical biological questions is a somewhat unusual one. However, this is the current opinion of many researchers working on the analogous processes of programmed cell death and apoptosis. Death of the organism is regarded as a tragic event, but at the cellular level it is, paradoxically, a prerequisite for life. Multicellular organisms have evolved specialized tissues that act in concert to enable the development and propagation of the organism. Every individual cell within each specialized tissue must function harmoniously with both its surrounding neighbours and the whole organism. It is the breakdown of these complex and integrated control systems at the cellular level, particularly loss of tissue homeostasis that is responsible for the onset of many human diseases.

Loss of tissue homeostasis can occur through over-proliferation of cells or inappropriate differentiation of cells. Significantly, the inappropriate death or survival of cells can also disrupt tissue homeostasis, a process that has hitherto been often overlooked. The study of apoptosis has revealed that the default-state of all cells in multicellular animals is death, with survival, proliferation, and differentiation all requiring positive inputs (1). Failure of this in-built suicide mechanism has drastic consequences. Aberrant surviving cells are implicated in the neoplastic process and the inappropriate death of cells can lead to, amongst others, neurodegeneration (2, 3).

Over the past twenty years research into programmed cell death has increased our knowledge of both the normal and pathological processes that can occur within tissues. This chapter serves as a broad introduction to the complex genetic and biochemical pathways of death that have been identified and which so preoccupy current apoptosis devotees.

2. Historically speaking

Wyllie, Kerr, and Currie initially used the word apoptosis in 1972 to describe a novel form of cell death (3). Cells were observed to die in a manner morphologically distinct from necrotic cell death (where the cell membrane ruptures and inflammatory cells are recruited to the scene). The 'apoptotic' deaths followed a defined

morphological sequence that did not result in loss of membrane integrity, or in an inflammatory response. Moreover, these cells were disposed of rapidly by their nearest viable neighbour or passing phagocytic cell. The same morphological features had been described much earlier by developmental biologists and referred to as 'programmed cell death' (4, 5). Here, biologists had observed the neat deletion of cells that were no longer required within the developing embryo. However, the significance of this type of cell death was not realized until its description in pathological and physiological situations occurring in the adult animal. From these studies it is evident that apoptosis or programmed cell death is a regulated form of cell death that does not normally activate the immune response. Thus, throughout the life of multicellular animals, cells are regularly lost by apoptosis (6). These cells may no longer be required, or may be damaged in some way. Moreover, such cells are silently removed from the organism allowing the tissue to function as normal.

Programmed cell death or apoptosis occurs in all multicellular animals and is studied in a wide variety of animals. Indeed, many of the features of apoptosis are evolutionarily conserved in both invertebrates and vertebrates. Much of the data on the genetic regulation of apoptosis has come from the nematode worm *Caenorhabditis elegans* (7), whereas many of the biochemical pathways have been described in mammalian cell systems (8). These advances in the understanding of cell death were stimulated by the morphological description of apoptosis.

2.1 Morphological aspects

The differences between necrosis and apoptosis have been reviewed many times before hence only the salient points will be addressed here. For a more detailed description see refs 2, 9, and 10.

2.1.1 Evidence of death

Overall, necrotic and apoptotic cells are quite different. Both forms of cell death involve a characteristic number of morphological changes that can be used to classify dying cells as either apoptotic or necrotic. Necrotic cells are characterized by an overall increase in cellular size, with little change in the chromatin initially (10, 11). Organelles within the cytoplasm become disorganized and mitochondria begin to undergo distinct changes, including the accumulation of lipid-rich particles within the mitochondria and swelling, with the inner mitochondrial membrane shrinking from the outer. The cyto-architecture of the cell is lost at later stages with general release of proteases, nucleases, and lysosomal contents. The chromatin becomes flocculent and then disperses. Rupture of the cell membrane occurs leading to release of the cellular contents and pro-inflammatory cytokines leading to the recruitment of monocytes and macrophages to the site of death (see Fig. 1).

Apoptosis also follows a characteristic but distinct number of sequential morphological changes (3). Apoptotic cells shrink in size and exhibit marked alterations in their chromatin structure at an early stage. The chromatin becomes highly condensed within the nucleus and can appear concentric with the nuclear

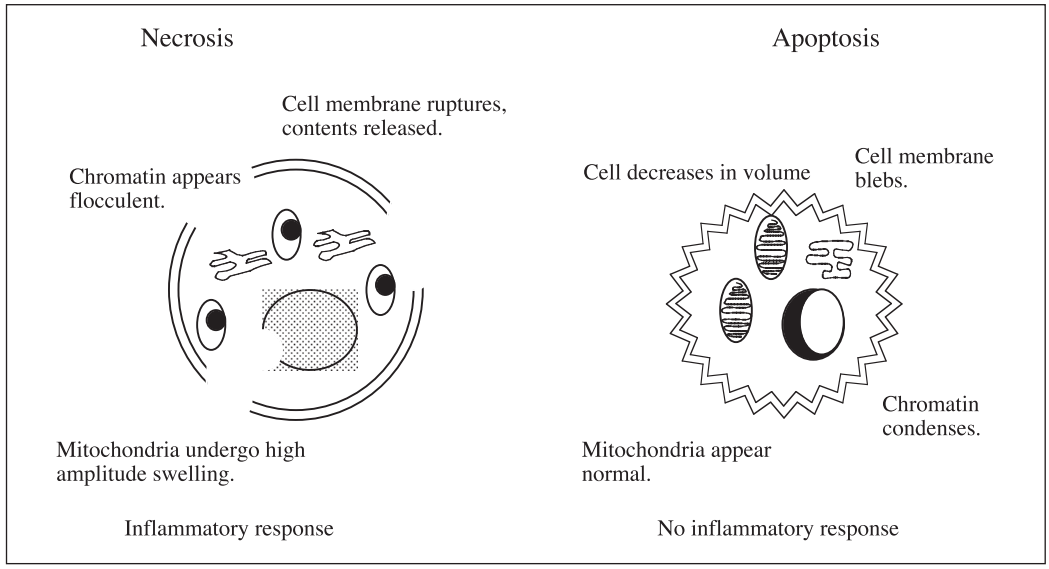

Fig. 1 Cells undergo distinct morphological changes when they progress through either necrosis or apoptosis. Necrosis is characterized by cell swelling and disruption of cellular organelles. Necrotic cells lyse releasing their contents and trigger an immune response. In contrast, apoptotic cells shrink, undergo membrane blebbing, and chromatin condensation. The cellular contents are retained and the cells are rapidly phagocytosed by viable neighbours or professional phagocytes.

membrane. The organelles within the cytoplasm remain intact and show little change apart from some swelling of the endoplasmic reticulum. Externally the cell appears to boil as the membrane becomes convoluted, this phase is often referred to as 'blebbing' and is one of the earliest morphological manifestations of imminent death (see Fig. 1). Cells *in vitro* often shed apoptotic bodies, which are membrane-bound vesicles containing intact cellular organelles and sometimes condensed chromatin. Whether or not apoptotic bodies are shed *in vivo* is still unclear. Data from time lapse video microscopy suggests that apoptotic cells can be phagocytosed prior to the shedding of apoptotic bodies. In cases where phagocytosis is delayed, breakdown of the cell into apoptotic bodies is more likely to occur (12).

Several of the morphological changes that an apoptotic cell passes through are used in the laboratory to identify apoptotic cell death. One of the best markers is still the condensation of the chromatin, observed either by light, fluorescent, or electron microscopy (13). Other biochemical methods used to identify apoptotic cells are well documented (see ref. 14) and will not be discussed here.

2.1.2 Time of death

In real time, cells can enter apoptosis and be phagocytosed within an hour, making their appearance very transient indeed. Death is also stochastic; apoptotic stimuli do not tend to induce death in all cells at precisely the same moment. Therefore, when observing a tissue section at a specific time after an apoptotic stimulus very few

apoptotic cells will be observed (13, 15). Thus, evidence of apoptotic cell death is hard to determine from one time point and is one of the reasons why the importance of cell death was initially overlooked. Even a tissue that is essentially involuting may only appear to have 5% of cells in the apoptotic state at any one time, thus masking what is in fact a high level of death. One of the reasons why apoptotic cells are not often encountered in tissue sections is due to their rapid clearance by phagocytosis.

i. Disposal of the body

Despite all of the morphological changes that distinguish an apoptotic cell, perhaps one of the most important aspects of apoptosis is the rapid phagocytosis of the dying cell (9, 16). The physiological changes upon the cell surface that 'label' the cell as apoptotic and ready for phagocytosis occur very early on in the process. Current models suggest that this enables the clearance of the dying cell prior to the onset of secondary necrosis and membrane rupture, thus eliminating the involvement of the immune response (16). Invasion of a tissue by inflammatory cells can significantly disrupt the function of that tissue and hence needs to be avoided where possible (17). Importantly, the apoptosing cell does not need to attract the attentions of a passing professional phagocyte; instead it can alert its nearest viable neighbour and have them dispose of the body (18). Failure to remove an apoptotic cell will elicit an immune response since that cell will undergo secondary necrosis and lyse. In some tissues where the onset of apoptosis is acute and there are many dying cells phagocytosis is inefficient and many apoptotic cells undergo secondary necrosis. It is in these tissues that death is still classed as being necrotic, overlooking the fact that death may well have been apoptotic originally (19). This, even today, still leads to arguments about the relevance of apoptotic death. Interestingly, these uneaten secondarily necrotic cells elicit release of a specific cocktail of inflammatory mediators from the phagocytosing macrophage, the contents of which is dependent upon the time past the cells 'eat-me-by' date (20, 21). Thus, only where there is massive apoptotic cell death, inefficient phagocytosis, and secondary necrosis is the inflammatory response fully involved (16, 21).

Although treated somewhat superficially by many researchers in the field, the removal of apoptotic cells is a critical process. It is not yet clear how detrimental failure to remove apoptotic cells is to a tissue and this is one area of research that is only now coming to the fore (21). Significantly, six known genes regulate the phagocytosis of dead cells in the nematode *Caenorhabditis elegans* (see Chapter 2). Conservation of the cell death machinery is evident throughout evolution, although a greater number of genes are involved in mammalian cells (22–24). The elaborate nature of the phagocytic signals in mammalian cells is still being deciphered and is excellently reviewed in refs 25–27.

3. Where does apoptosis occur?

Apoptosis occurs in all multicellular organisms. Death of cells is seen mainly during development and is important primarily for deleting unwanted cells (6). There are

many examples of where programmed cell death occurs and this has been the subject of several previous reviews (3–6). In general, cells are deleted when sculpturing tissues within the body such as formation of the digits (deletion of the inter-digital cells) (28). Cells are also deleted to remove structures that are no longer required, involution of the tadpole tail being the most cited example. Deletion of cells is also a good mechanism for precisely matching the number of cells required for a particular function. In the nervous system many more neurons and oligodendrocytes are produced than are required. Up to half are eliminated since they fail to make productive interactions with target cells (29). Finally, potentially harmful cells are deleted. A good example is the deletion of cells in the thymus during positive and negative selection (30). Thymocytes that have functionally inactive receptors, or produce potentially self-recognizing receptors undergo programmed cell death.

Programmed cell death is therefore most evident during multicellular animal development. However, apoptosis also occurs sporadically in all tissues throughout life and is a normal everyday occurrence in tissue turnover.

3.1 Primitive death

One of the most comprehensively studied examples of programmed cell death is that which occurs in the nematode *Caenorhabditis elegans* (7, 22, 31). The development of each *C. elegans* worm is invariant, 1090 cells are generated of which 131 die. The death of these 131 cells is regulated by a core of approximately 13 genes that either specify the death of the cell, carry out its suicide, or phagocytose the cell and dispose of the body (see Chapter 2). Many of these genes have been conserved throughout evolution and are functionally and structurally similar to their mammalian counterparts (Fig. 2). Hence, the function of these genes in the relatively simple nematode can be extrapolated to identify genes of similar function in higher order animals.

Evidence for programmed cell death is also seen in primitive slime moulds such as *Dictyostelium discoideum* (32). When conditions are favourable these eukaryotic cells exist as free living amoeboid cells. When food is in short supply the amoeboid cells aggregate and form a fungal-like structure with a stalk and a fruiting body. The cells within the stalk die as part of a terminal differentiation programme and these deaths exhibit morphological changes similar to those seen in eukaryotic cells.

Cell death also occurs in plants and can be seen in the development of xylem, flowers, and ovules (33). It can also occur in the hypersensitive reaction to invading pathogens. Although the dying plant cells have some similar morphological features to apoptotic animal cells, they are not phagocytosed. Presumably the presence of a cell wall prevents this. The molecular mechanisms controlling this form of cell death have yet to be fully investigated, but it would be interesting if plants also have a genetically conserved cell death programme.

The widespread occurrence of cell death and its apparent conservation throughout evolution suggests that regulation of cell death is critical in the development of multicellular organisms. Many genes have now been shown to regulate various steps within the cell death pathway and form the basis of many of the chapters in this

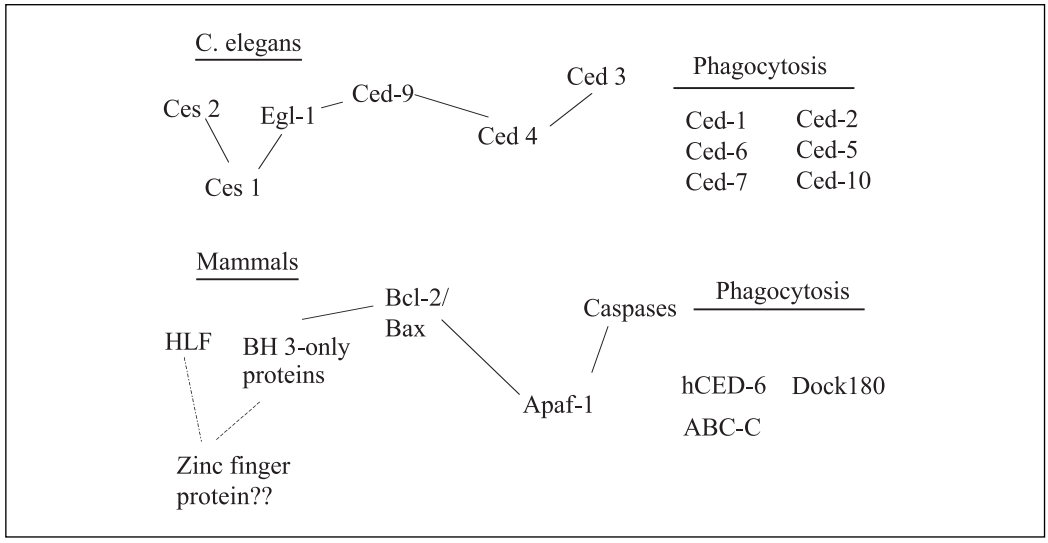

Fig. 2 Comparison of cell death pathways in nematode and man (22). Cell death is specified by Ces-1 and Ces-2 in *C. elegans* which appear to be transcription factors. Ced-9, the anti-apoptotic protein is inhibited by Egl-1, allowing activation of both Ced-4 and Ced-3. A similar pathway exists in mammalian cells with transcription factors at the start of the pathway (hepatic leukaemia factor, a basic helix-loop-helix protein), followed by the BH3-only members of the Bcl-2 family. BH3-only proteins, like Egl-1, antagonize the function of the Bcl-2-like proteins. This enables the activation of Apaf-1 (Ced-4) and caspases (Ced-3). Apoptotic cells are phagocytosed in both nematode and man and several homologous genes carry out this function in both animals.

book. However, what remains unclear is where these genes act to commit a cell to death. This commitment point is still a hotly debated topic, but for simplicity, genes can be thought of as acting in three basic phases; decision, sentence, and execution. The significance of each phase is outlined below.

4. The importance of regulated cell death

Prior to the discovery of apoptosis, cell population numbers were known to be controlled by differentiation, proliferation, and senescence. The discovery of genes that actively prevented apoptosis and the demonstration that inhibition of protein and RNA synthesis in some cell types delayed apoptosis suggested that this form of cell death was regulatable and therefore potentially important in cell population control (34–36). Indeed it is now clear that a large number of proteins act to direct whether a cell lives or dies. Moreover, this has led to the idea that all cells are programmed to die unless prevented from doing so. Thus, death is the default state of all cells.

The various fates of differentiation, proliferation, death, and senescence control cell population number (Fig. 3) (19). For cells that lose the capacity to undergo apoptosis in response to physiological stimuli the consequences can be quite dramatic. For example, one gene *bcl-2*, the first of a now large family of apoptosis-

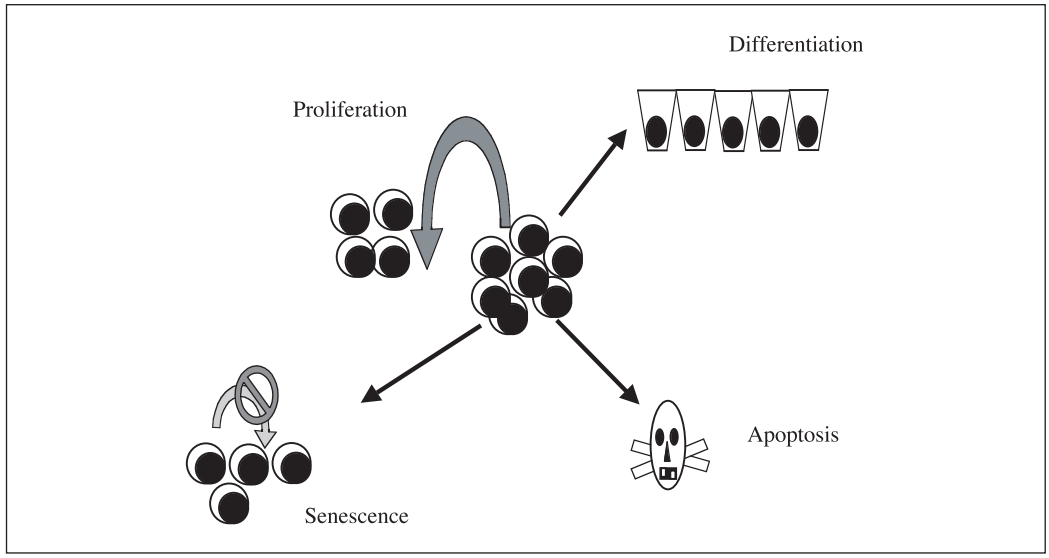

Fig. 3 Tissue homeostasis is maintained by the opposing functions of differentiation, senescence, proliferation, and death. Loss of any one of these pathways is implicated in the development of human disease.

regulating genes to be identified, causes excess accumulation of B cells when overexpressed in the B cell lineage in Eμ-*bcl-2* transgenic mice (37). These cells are not at first detrimental to the animal, however, the increased number of cells substantially increases the risk of a cell attaining a secondary and/or tertiary mutation that facilitates the clonogenic outgrowth of that cell. Thus, *bcl-2* transgenic mice develop follicular lymphoma at 12 months of age. The inappropriate survival of cells therefore, even for transient periods, is deleterious to the maintenance of tissue homeostasis.

Conversely, too much cell death can also have adverse effects. Cells can be lost from tissues due to the presence of non-physiological apoptotic stimuli or due to an over-sensitization to a particular physiological stimulus. A good example of a non-physiological stimulus of cell death would be the loss of neurons in Alzheimer's. Here cells amass aberrant forms of a specific protein that accumulates producing the characteristic disease-associated plaques that are toxic to the cell (38).

Thus, perturbations within the cell death pathway can have very profound effects on tissue physiology. However, since apoptosis is a regulated form of cell death, comprehension of the pathways that act to control whether a cell lives or dies should reveal novel targets for disease intervention. Much is now understood of the mechanisms involved in regulating cell death in several different species. Moreover, the complex system that exists in mammalian cells is paralleled by the less complex nematode and *Drosophila* cell systems. The combined research into all these animal models has identified a number of complex signalling networks involved in the apoptotic pathway.

4.1 The decision phase

One of the problems with working on a phenomenon that was initially described based upon its morphological characteristics is that many gene products do not affect these morphological changes. Indeed, the appearance of morphologically apoptotic cells characterizes the final stage of the process of regulated cellular death. The first arbitrarily assigned stage, the decision phase, is one not defined by any morphological criteria. Instead, it defines the phase that is presumed to occur when external and internal pro- and anti-apoptotic information is integrated within the cell. Acting within this phase is a whole host of known and novel gene products (see Fig. 4). Many of these gene products and their roles within the apoptotic pathway are discussed in depth throughout this book, so the following represent a brief resume only.

4.1.1 Apoptosis and genes that regulate the cell cycle

Many of the genes that are primarily associated with cell cycle regulation are also fundamentally important in the control of cell death. Products of the c-*myc*, *E2F*, c-*fos*, *ras*, and c-*abl*, genes have all been shown to exert some form of control over a cells capacity to commit suicide (39). In particular, the role of c-Myc in regulating both cell proliferation and death has underscored the intimacy of these two processes (39).

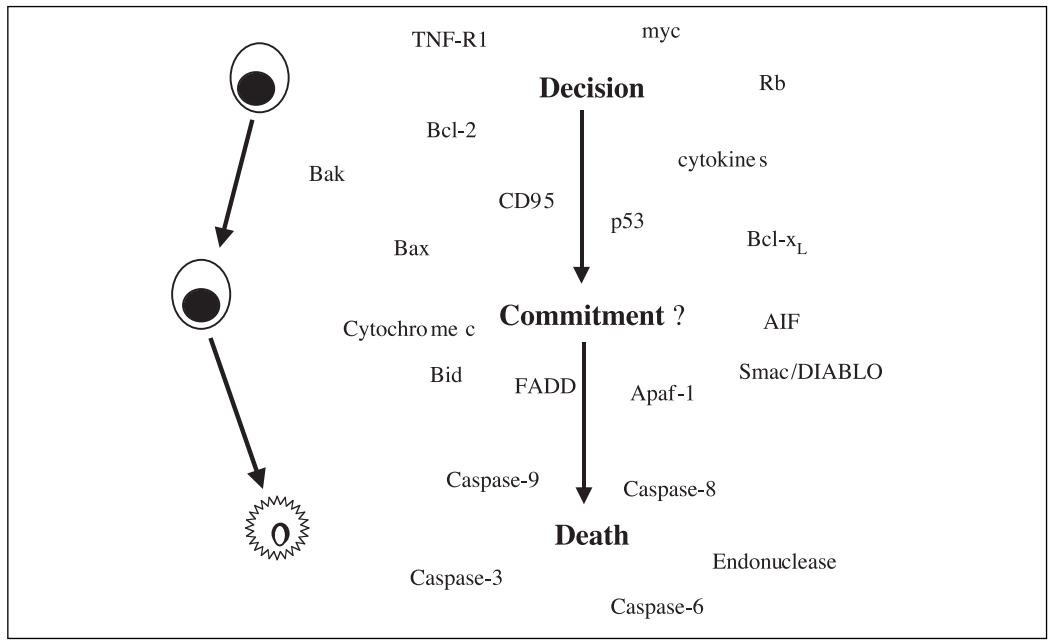

Fig. 4 Genes acting in the apoptotic pathway. The cell death pathway can be arbitrarily divided into three phases: decision, commitment, and death (execution). Where genes act within this pathway appears to be dependent upon the stimulus and the cell type being studied. Central to the understanding of apoptosis regulation at present is identifying which factors act to commit a cell to death.

The cell cycle regulatory proteins p53 and Rb can also influence a cells capacity to survive (40). Loss of Rb function produces excess apoptosis during development and slow growth and transformation in tumour cell models (41, 42). Conversely, loss of p53 enhances cellular survival in the presence of DNA damage and aides tumour progression (43, 44). Loss of both p53 and Rb leads to enhanced tumour progression, again suggesting that loss of cell cycle regulation affects apoptosis (45).

It is initially counter-intuitive that gene products which regulate proliferation can also regulate the opposing demise of the cell. However, in the context of multi-cellular, large, long-lived animals this is a critical 'fail-safe' mechanism. In terms of the neoplastic process, potentially the most dangerous cell within a tissue is one that replicates its DNA and then divides it between two daughter cells. Any mutation that confers a growth advantage could lead to tumour formation. However, if a cell that has the capacity to divide is also more sensitive to death, then any cell harbouring an increased capacity to replicate will also be more likely to die (46). Recent evidence widely supports this hypothesis demonstrating that cells with oncogenic lesions are more sensitive to death stimuli (reviewed in Chapter 8 and ref. 47). Moreover, genes that enhance survival, such as *bcl-2* are also cytostatic, making a long-lived cell less likely to divide (48, 49). The combined lesions of enhanced survival and proliferation are highly tumorigenic (50).

4.1.2 The Bcl-2 family

bcl-2 is often thought of as the first gene identified that primarily acted to enhance cellular survival in mammalian cells (51). Like its nematode homologue *ced-9*, *bcl-2* can suppress apoptosis in response to specific stimuli. Moreover, its inappropriate expression in cells is associated with enhanced survival capacity which can lead to tumour formation or autoimmune dysfunction, depending the cell lineage affected (52, 53) (see Chapter 5). Other Bcl-2-like proteins exist within mammalian cells, some of which are anti-apoptotic and others, like Bax, Bak, Bad, and Bik that are pro-apoptotic (Fig. 5). These proteins are found in many compartments within the cell, but it is their mitochondrial association that has recently been closely investigated (54). Bcl-2 family members appear to regulate the release of cytochrome *c* from mitochondria, which acts in concert with specific downstream factors (caspases) to induce apoptosis (55) (see Chapters 4, 5, and 6). In addition, members of the Bcl-2 family interact with one another and regulate each other's function (56). Binding of a pro-apoptotic member to an anti-apoptotic one can result in the loss of the anti-apoptotic function and lead to the death of the cell. Therefore, the balance of pro- and anti-apoptotic members of the Bcl-2 family regulates an important step in the decision to live or die.

4.1.3 Death receptors

The discovery of antibodies that specifically trigger the death of tumour cells lead to the identification of cell surface receptors responsible for this effect (57). These receptors are members of the tumour necrosis factor receptor (TNFR) superfamily (58). Binding of specific ligands to these receptors causes their aggregation and

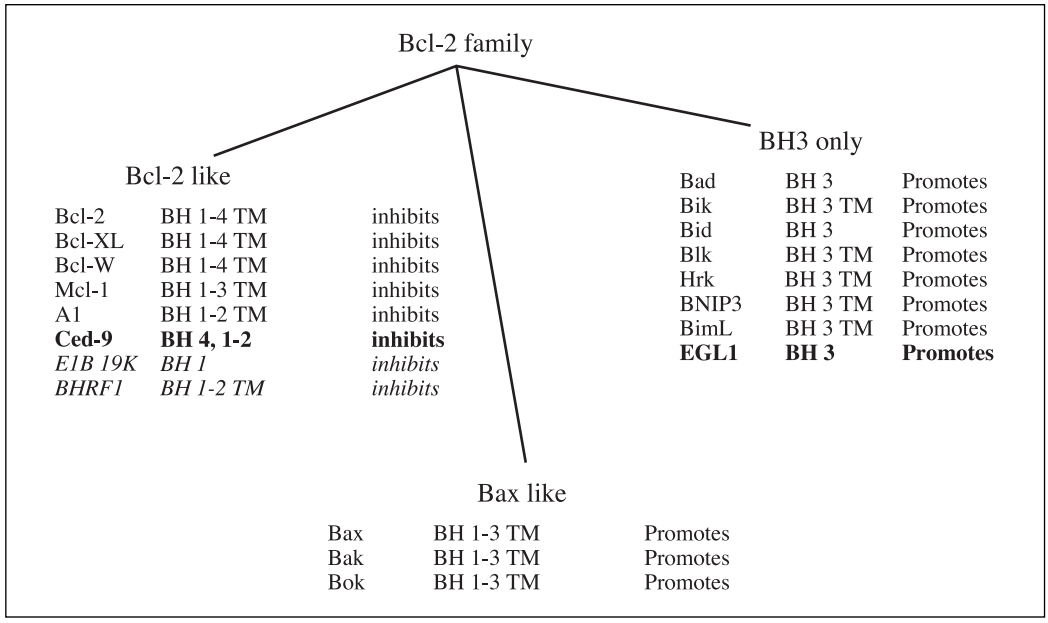

Fig. 5 Members of the *bcl-2/ced-9* gene family (after ref. 112). Members of this family can be split into three groups. The first is defined by proteins that contain four regions of homology (Bcl-2 homology-BH) and/or are anti-apoptotic in function. The second contains Bax-like proteins that do not have the 'protective' BH4 domain and activate apoptosis. The final group are BH3-only proteins that all act to activate apoptosis. Some, but not all members of the Bcl-2 family contain a transmembrane domain (TM) allowing localization to a number of membrane targets within the cell. (Bold indicates homologous genes from *C. elegans*, italics indicate viral homologues.)

activation. To the intracellular domains of these activated receptors bind several downstream adaptor proteins, some of which can activate the caspase family of proteases that carry out the systematic degradation of the cell (Fig. 6) (59–61). Other adaptor proteins initiate other signalling pathways within the cell; thus the cells will not always undergo cell death upon receptor activation (62). Death can be prevented by the presence of specific anti-apoptotic proteins that prevent the downstream caspases binding to the receptors, or in some cells the presence of survival factors such as Bcl-2 or survival cytokines will prevent cell death (63) (see Chapter 8). The importance of this family of death receptors in the immune system is particularly well understood. However, many other cell types express these receptors and moreover, they express more than one family member, giving rise to a complex network of cell death regulation. It is clear however that these receptors can either induce death or in some cells proliferation or differentiation, making them part of the decision process.

4.1.4 Survival cytokines

Cytokines have many different effects within cells that are generally cell type specific. One effect mediated by cytokines is cell survival; withdrawal of a specific survival cytokine from cells induces apoptosis (64, 65). In the presence of specific cytokines

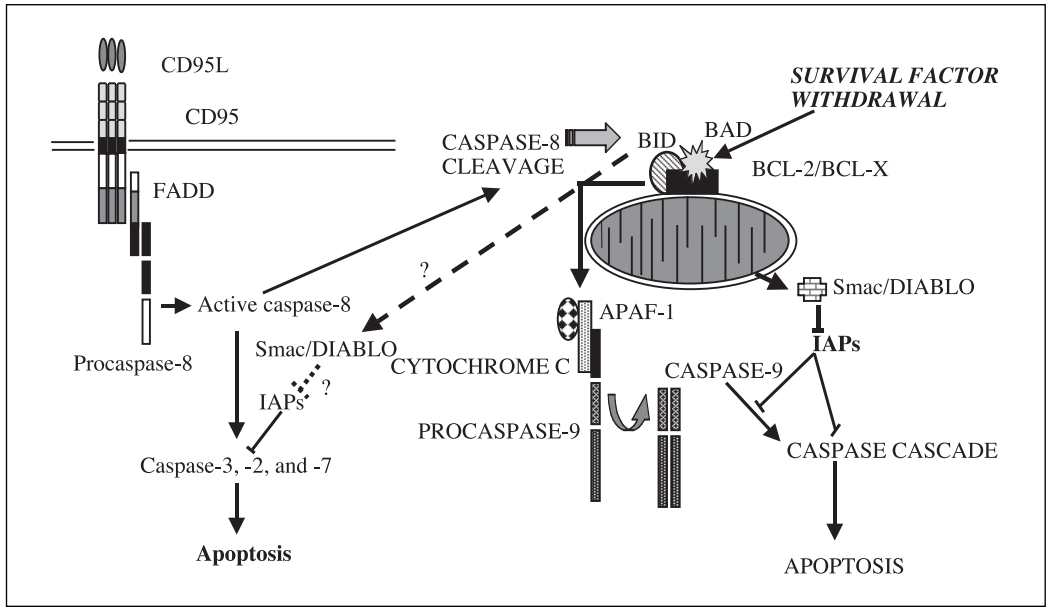

Fig. 6 Two pathways of apoptosis. Depicted are two pathways through which caspases can become activated. The first is the death receptor pathway involving activation of the CD95 receptor via its ligand CD95L. This triggers the binding of FADD to the receptor, which in turn recruits caspase-8. The presence of many caspase-8 molecules in close proximity leads to autoactivation. Once active, caspase-8 cleaves downstream caspases, such as caspases-3, -2, and -7, triggering the death of the cell. Alternatively, caspase-9 can be activated by release of cytochrome *c* from mitochondria. Cytochrome *c* along with ATP and Apaf-1 forms a complex to which caspase-9 molecules bind. Due to the close proximity of caspase-9 molecules in this complex, autoactivation of caspase-9 occurs. Active caspase-9 cleaves other downstream caspases again triggering apoptotic changes within the cell. These two pathways are linked by caspase cleavage of BH3-only proteins, such as Bid, that alter Bcl-2 family proteins at the mitochondrial membrane leading to release of cytochrome *c*. Inhibition of caspases by the IAPs is overcome by release of Smac/DIABLO from the mitochondria.

cells can survive, even if the cell happens to receive a pro-apoptotic stimulus (Fig. 6). The downstream pathways triggered by survival cytokine binding have been studied in many cell types in response to factors such as platelet-derived growth factor (PDGF), nerve growth factor (NGF), interleukin-3 (IL-3), and insulin-like growth factor 1 (IGF-1). IGF-1, for example, potentially activates both pro- and anti-apoptotic pathways, via Ras activation. The pro-apoptotic pathway is mediated by Ras-Raf signalling and the anti-apoptotic pathway by p110-mediated Akt/PKB signalling (66). Akt is a very important anti-apoptotic protein that acts to inhibit cell death at various stages within the apoptotic pathway (67–69) (see Chapter 9). Thus, the presence of survival cytokines bypasses the default-state of all cells, which is death.

4.1.5 Summary

Overall there are many genes that can influence a cells' likelihood of death. Many of these genes feed into a common pathway that involves release of cytochrome *c* from

mitochondria and the activation of caspases. The release of cytochrome *c* from mitochondria is an active area of research at the current time primarily because it is one of the very early changes that occurs in almost all apoptotic cells and maybe involved in the commitment of cells to death (70).

4.2 The commitment phase

Which components of the apoptotic signalling pathway act within the commitment phase is a controversial topic. The fundamental question is precisely which molecular changes occur when a cell is condemned to death? If these particular changes can be identified then it may be possible to regulate both cell death and cell survival very effectively. Ideally, new pharmacological targets will be found that can induce death in cancer cells or prevent neuronal death in Alzheimer's.

What are the candidates for this phase of cell death? At present no known molecule that acts in the cell death pathway in mammalian cells is required absolutely for cell death to occur in all cell types. Thus, there is no one golden molecule to cure all apoptosis-associated ills. Perhaps this is not too surprising given the biological havoc that could ensue upon the mutation of this one molecule. Instead it appears that the commitment phase will vary depending upon the stimulus and the cell type concerned. A good example of this has recently been provided by the generation of Apaf-1-deficient animals (71). *apaf-1* is the mammalian homologue of *ced-4*, a gene required in the nematode for *all* somatic cell deaths to occur (72). *apaf-1*-deficient mice exhibit perinatal lethality. The defects that these mice display are mainly within the developing nervous system. Cell death is critical during neuronal development. Many more neuronal cells are produced than are needed and those that do not make connections with other neurons do not receive sufficient anti-apoptotic stimuli. Thus these cells are lost silently by apoptosis during embryogenesis. In Apaf-1-deficient animals, these cells are no longer susceptible to developmental death and remain viable. As a result, areas of the fore- and hindbrain are too big to be encapsulated by the skull bones and mice are born with neuronal protrusions. Interestingly, they also do not lose the inter-digital cells between the digits and are born with webbed paws. Apaf-1 is important in the activation of downstream caspases and the onset of the execution phase of cell death. The lack of cell death in developing neurons suggests that Apaf-1 is required for the commitment to death. Alternatively, one could argue that Apaf-1 acts in the decision phase and absence of Apaf-1 produces a potent survival signal. However, the absence of Apaf-1 in these animals does not prevent death receptor-induced cell death. Moreover, no other tissue abnormalities have been described suggesting that other death pathways are functional within these animals. Thus, Apaf-1 is not required for all apoptotic cell deaths.

Release of apoptogenic factors from mitochondria is also a prime candidate for regulation of the commitment point. Several apoptosis-inducing agents are released from mitochondria including cytochrome *c* and apoptosis-inducing factor (AIF) (73) Evidence suggests that release of cytochrome *c* however, does not necessarily induce

apoptosis. Cells injected with cytochrome *c* can remain healthy in the absence of pro-apoptotic signals (74). Thus, release of factors from the mitochondria may not be the point of no return.

The recent description of a novel molecule termed either Smac or DIABLO has exemplified the role of mitochondria in the cell death process (75, 76). Smac/DIABLO also resides within the mitochondria and is released at or around the same time that cytochrome *c* release occurs, through mechanisms which are at present unclear. Smac/DIABLO is able to bind to a specific class of anti-apoptotic proteins termed IAPs (inhibitors of apoptosis). IAPs were initially described in baculovirus, but have since been characterized in nematodes, *Drosophila*, and mammals (77). IAPs have several functions, but it is their ability to inhibit active caspases that is the primary target of Smac/DIABLO (78). Smac/DIABLO binds IAPs that are bound to active caspases (-9 and -3 being prime examples) and removes them, preventing inhibition of the caspase cascade (see Fig. 6). This may explain why microinjection of cytochrome *c* does not always trigger apoptosis, since without the release of Smac/DIABLO from the mitochondria, caspases may well remain inactive due to the presence of bound IAPs (79). Thus, release of factors from the mitochondrion is certainly important for the activation of the caspase cascade. However, caspase-8 (an upstream caspase) is not targeted by IAPs (80). Hence activation of the CD95 receptor, which activates caspase-8, may not be influenced by the presence of Smac/DIABLO, again suggesting the role of this protein is context dependent (79).

Overall, which cell death molecules are included in the decision and commitment phases of cell death depends upon the model system being used and this is reflected within the chapters of this book.

4.3 The execution phase

The execution phase can be used to define the point at which all of the morphological changes that are associated with apoptosis become apparent (81, 82). The critical mediators of this phase of apoptosis are the caspases (83, 84). Caspases are proteases that cleave exclusively after aspartate residues and have a conserved active site, QACxG. There are 14 known caspases in mammalian cells with homologues in both nematode and *Drosophila* (85, 86). In mammalian cells, caspases can be broadly grouped into upstream or downstream caspases (Fig. 6). The upstream caspases are activated first, by a number of mechanisms, and once they are active they cleave and activate the downstream caspases. The caspases act in a cascade and cleave specific substrates that aid the specific breakdown and packaging of the dying cell for phagocytosis (87). Not all caspases are critical for cell death to occur (88). Indeed, in fibroblast cells treated with a broad-spectrum caspase inhibitor, the commitment to death is not suppressed, but the morphological changes of apoptosis are significantly delayed (89). However, both caspase-3 and caspase-9-deficient animals have severe abnormalities within the nervous system, that, like *apaf-1* null mice, is due to the survival of excess neuronal cells (90). Thus, both upstream and downstream caspases (-9 and -3 respectively) are critical for death to occur during development of the

nervous system. In some tissues, therefore, certain caspases could be classified as acting during the commitment to cell death, whereas in others they are not required for commitment to occur. This again underscores tissue-specific differences in apoptosis regulation.

4.4 Summary

From the initial description of an intriguing morphological phenomenon has come the description of a biochemically complex, genetically regulated mechanism for the elimination of unwanted or damaged cells. Research into apoptosis is at a particularly interesting stage in that many more genes are now known to regulate death, but not all seem likely to act in the same way in every cell. Particular genes may be critical for one apoptotic signal, yet of no use for another. This maybe one reason why cells appear never to lose the capacity to die. They may exhibit resistance to one or more cell death triggers, but if pushed hard enough or with the right stimuli, cells which are apparently resistant will undergo apoptosis. So for each gene mentioned above, its role in tissue-specific death must be closely analysed before one characterizes its function within the death pathway. The arbitrary assignment of genes to the three 'phases' of apoptotic cell death is helpful when theoretically considering which gene product functions where, but the boundaries between these assignments must remain dynamic. This is a reflection of both the differences between tissues as mentioned above and the rapid progress that is made in this field. Researchers looking to find genes that act in apoptotic pathways have identified many novel genes. It is then often taken for granted that 'apoptosis regulation' is the sole function of the identified gene. However, many of the genes that function in this pathway are active in many other cellular processes and their initial role in apoptosis may turn out to be a small one indeed.

5. Models of apoptosis

Many cellular models are now used to study the process of apoptosis. This includes simple organisms such as *C. elegans* and *Drosophila* and the much more genetically complex models of mouse and man.

Animals such as *Drosophila* and *C. elegans* have been very valuable in understanding the genetic regulation of death, especially in the case of *C. elegans* whose genetic lineage is completely mapped. The function of all the cells within this organism is also known so cell type-specific functions of genes can be closely investigated. Thus, with the conservation of the death machinery throughout evolution, there is still much to be gained from genetic studies in the nematode (22). *Drosophila* is a useful system since its is amenable to the study of both genetic and biochemical consequences of gene expression (91). There is, for example, much scope within this animal for investigating the role of different caspases during cell death. However, it is interesting to note that the genes that regulate death within *Drosophila* (*reaper*, *hid*, and *grim*) have no homologues in either *C. elegans* or mammalian cells

(92). However, one function of Reaper, Hid, and Grim is to target and inhibit IAPs, suggesting some functional similarity between these *Drosophila* proteins and Smac/DIABLO (78, 79, and Chapter 3). A major limitation of both *C. elegans* and *Drosophila* is that neither suffers from the problems faced by long-lived organisms. Loss of cell death in *C. elegans* does not lead to tumour formation. Indeed, worms with excess cells appear normal (although recent data does suggest some loss of vigour). Thus, the tight regulation restricting cell growth in higher order organisms may not exist in these lower order animals. These caveats apart, the study of cell death in these animals is highly productive as demonstrated in Chapters 2 and 3.

The use of transgenic and knockout mice to study the consequences of aberrant apoptosis *in vivo* is also very valuable since such studies have elicited some unexpected findings that would not have been predicted from *in vitro* studies (93). One such example is the generation of Rb null mice (94–96). Given the important role of Rb in restricting cell cycle progression, one may have predicted that its loss would allow for unrestricted cell division resulting in an animal that was highly susceptible to cancer development. However, Rb null mice are non-viable due to massive apoptosis occurring in the central nervous system and tissues associated with red blood cell production. This is principally because these cells are unable to exit cell cycle and respond to the changing cytokine environment. Without appropriate survival factors these cells die, underlining the connection between cell proliferation and cell death.

The recent generation of mice that specifically express the oncogene c-*myc* in the skin has shown that the importance of apoptosis in the fight to suppress neoplasia depends very much on how cell numbers are controlled within a specific tissue (97). Overexpression of c-Myc in fibroblast cells *in vitro* makes them much more susceptible to cell death (98). The prediction would be that when expressed inappropriately in the skin, the cells would proliferate and also undergo apoptosis. However, although proliferation was seen in these transgenic mice, apoptosis was not. One reason for this result maybe that because skin cells are normally sloughed off once terminally differentiated, apoptosis is not required to remove the excess cells produced by Myc-induced proliferation. Instead excess cells are simply shed in greater numbers. Conversely, mice expressing a Myc transgene in β-islet cells of the pancreas develop diabetes due to Myc-induced apoptosis and loss of this population of cells (99). Thus, transgenic models are important for understanding the particular function of a pro- or anti-apoptotic gene within a particular tissue.

Another way in which pro- and anti-apoptotic proteins have been investigated is through use of cell-free systems (8, 83, 100, 101). These are generated from either mammalian cells treated with a pro-apoptotic stimulus or from using *Xenopus* egg extracts (see Chapter 7). The use of cell-free systems enables the biochemical investigation of proteins such as caspases and Bcl-2 family members. Cell-free systems have identified some of the critical pathways involved in regulating cell death, such as cytochrome *c* release and the presence of Apaf-1 and caspase-9 complexes (72). Such biochemically-based systems greatly aid the interpretation of data from both invertebrate and vertebrate models of death. However, they are

somewhat limited to investigating the later stages of apoptosis such as caspase activation. Extracts derived from *Xenopus* do allow the study of several 'upstream' factors such as Bcl-2 family members (101, 102) and for the analysis of proteins from other animals involved in cell death (*reaper* from *Drosophila* being one such example).

Other models systems such as yeast, bacterial pathogens, viruses, and plants are also used to study cell death (33, 103–105). The study of viral encoded genes has identified several anti-apoptotic genes that are also conserved within mammalian cells (105–109). However, the degree to which the cell death machinery is conserved in yeast (110) and plants (111) has yet to be fully established.

Overall there are many models in which cell death is studied, all contributing their own special viewpoint on how cellular suicide is regulated.

6. Future work

Apoptosis is regulated by many genes that act in concert to ensure the demise and disposal of the condemned cell. At all of the stages of apoptosis discussed above, there are genes that act to induce and genes that act to suppress cell death. This sets up a very complex web of regulation befitting for a process that is critical in the maintenance of tissue homeostasis. Mutation of the cell death pathway is never absolute in that, although more resistant to specific triggers of apoptosis, cancer cells, for example, never completely lose their ability to undergo apoptosis. Thus, the goal of the apoptosis researcher is two-fold. First to identify a precise mechanism that regulates cell death within their chosen system, and secondly, to identify the key components within that system that will facilitate intervention in this process.

The following chapters review the critical genes involved in the regulation of apoptosis, their associated biochemical processes where known, and the diseases in which mutation of these genes is thought to be important. They provide a basic overview of research in apoptosis as it stands at the current time and review some of the controversial aspects within the field.

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