Why we live and why we die

Programmed cell death, or apoptosis, is a tightly-controlled phenomenon that is important in many pathological processes. Several important regulators of apoptosis have now been identified and can be targeted for manipulation.

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Although physiological cell death in tissues has been recognized for over a hundred years, it was only with the careful and detailed characterization of apoptosis by Kerr, Wyllie, and Currie [1] some 25 years ago (reviewed in [2–4]) that its importance as a fundamental biological process began to emerge. What has pivoted apoptosis into the centre stage of cell and molecular biology over the past two years has been the identification of genes that are responsible for regulating cell viability. Many of these genes are old friends;they have previously appeared in several different guises, as oncogenes, tumour suppressor genes, cell-cycle regulators, growth factors and proteases. As a consequence, although the biology of apoptosis is largely virgin territory, many of its regulatory players have already been subjected to extensive investigation and characterization. Modulation of apoptosis therefore represents an unparalleled opportunity for the development of new pharmacological agents that might allow an improved understanding of the control of cell death, and how death is essential to life.

Role of apoptosis in normal and pathological biology

Mammalian programmed cell death, often called 'apoptosis,' was identified as a distinct process by virtue of the discrete series of morphological changes exhibited by cells undergoing programmed cell death [4]. Apoptotic cells shrink in volume, their surfaces undergo dramatic convolution and blebbing, and the affected cell then fragments into discrete apoptotic bodies which are rapidly phagocytosed by neighbouring cells. Apoptosis is also usually accompanied by degradation of cellular DNA which can be identified by its characteristic appearance as a ladder of nucleosome-sized fragments upon agarose gel electrophoresis. The whole process of apoptosis is very rapid, typically taking under an hour. Moreover, apoptotic debris is rapidly cleared from tissues by phagocytosis. The rapidity of the process means that the degree of apoptosis occurring in tissues is frequently underestimated. In many proliferating tissues, apoptosis is often the commonest fate of cells: in certain specialized organs such as the thymus over 95 % of all cells undergo apoptosis.

Thus, the balance of a tissue mass is maintained not only by the proliferation, differentiation and migration of cells but also, in large part, by controlled loss of cells by apoptosis. Not surprisingly, therefore, dysfunctions in the control of apoptosis are implicated in a wide range of pathologies. Lesions leading to suppression of apoptosis appear to be fundamental components of the carcinogenic process — many oncogenes are directly involved in regulating programmed cell death (see below). Inappropriate apoptosis is implicated in the pathology of neurodegeneration [5] immunodeficiency (innate and acquired through HIV infection) [6], secondary cell death following ischemia or stroke and various degenerative disorders [7]. Pharmacological control of apoptosis therefore provides new opportunities for therapeutic intervention in many diseases and new potential targets for rational and empirical drug development.

Control of apoptosis is complex and still incompletely understood. Nonetheless, several important regulators of programmed cell death have been identified that operate through known molecular interactions. This review will focus on some of the best-characterized regulators of apoptosis: the family of proteins that includes the product of the proto-oncogene *bcl-2*; the family of proteases homologous to the interleukin-1β converting enzyme (ICE), and a third family of proteins defined by interactions with the product of the proto-oncogene c-*myc*.

Control of apoptosis

An evolutionarily conserved pathway

An intrinsic cell suicide pathway appears to have arisen very early during multicellular evolution. The evolutionary conservation of the molecular machinery responsible for programmed cell death has greatly facilitated our understanding of apoptotic pathways in mammalian cells, because components of the apoptotic pathway in man are substantially homologous to those regulating developmental cell death in the genetically 'tractable' nematode worm *Caenorhabditis elegans*.

During development of the hermaphrodite nematode worm *C. elegans* 1090 cells are born, of which 131 die by programmed cell death. Two genes, *ced-3* and *ced-4*, are required for these developmental deaths, and if either of these genes is inactivated by mutation none of the 131 cells dies [8]. Another gene, *ced-9*, acts to suppress *ced-3*/*ced-4*-dependent cell death [9]: indeed, *ced-9* is necessary for the survival of most, perhaps all, of the cells in the worm, even those not normally subject to programmed death.

As in *C. elegans*, mammalian apoptosis is regulated by a dynamic balance between molecular processes that trigger it and opposing mechanisms that suppress it. The mammalian homologue of the nematode death-suppressor gene *ced-9* is the protooncogene *bcl*-2, first identified at the site of reciprocal t(18:14) chromosome translocations in human follicular B cell lymphoma. Indeed, human *bcl*-2 can functionally replace *ced-9* in *C. elegans* [10,11]. The nematode *ced-3* killing gene encodes a protein homologous to the interleukin-1β converting enzyme (ICE), a cysteine protease [12]. No mammalian homologue of the nematode *ced-4* gene is yet known, although the Ced 4 protein contains motifs that mark it as a Ca^{2+}-binding polypeptide.

Bcl-2 and ICE are each members of families of homologous proteins that share related functions. The Bcl-2 family includes the splicing alternatives Bcl-X_L and Bcl-X_S, Mcl-1, A1, Bax, Bcl-Y and the viral proteins BHRF1 (Epstein–Barr Virus) VG16 of Herpesvirus saimiri, LMW5-HL (African Swine Fever Virus) and $p19^{E1B}$ (adenovirus); for a detailed review, see [13]. Members of the Bcl-2 family fall into two functional groups. Bcl-2, Bcl-X_L, BHRF1, $p19^{E1B}$ and A1 all inhibit programmed cell death whereas Bax [14,15], Bcl-Y (T. Chittenden, personal communication) and the smaller splice variant of Bcl-X, Bcl-X_S [16], antagonize the anti-apoptotic activity of Bcl-2. Recent studies indicate that Bcl-2 and Bax form heterodimers, and it is via these heterodimers that Bcl-2 exerts its anti-apoptotic effects [14,15]. In contrast, Bax forms homodimers that may actually trigger apoptosis.

The ICE family of proteases so far comprises ICE itself, the mouse NEDD2 protein [17] (also called ICH-1_S [18]), ICH-1 [18] and prICE [19]. Several lines of evidence argue for the direct involvement of ICE or ICE-like proteases in the induction of apoptosis in mammalian cells. First, ectopic expression of ICE triggers apoptosis in Rat-1 fibroblasts [20] and expression of the mouse protein NEDD2 induces apoptosis in neurons [17]. Second, inhibition of ICE function by co-expression of the pox virus-derived ICE inhibitor CrmA [21] suppresses apoptosis of neurons following factor deprivation [22]. Third, the ICE-like protease prICE, which is specific for poly-ADP ribose polymerase (PARP), has been identified as the principal trigger in an *in vitro* model of nuclear fragmentation which shares many similarities with apoptosis in intact cells [19] .

ICE is an Asp-specific protease. Recent resolution of the crystal structure of ICE [23] shows that the regions of highest conservation between human ICE and the product of the nematode *ced-3* gene lie in areas that appear to be important for the proteolytic activity of ICE. This supports the notion that *ced-3* also encodes a protease and, furthermore, argues that the critical apoptotic triggers, the targets of these proteases, might also have been conserved through evolution. The idea that specific proteases initiate an intracellular cascade resulting in apoptosis is reinforced by the fact that the natural killer cell protein fragmentin 2, which also triggers apoptosis when introduced into target cells, is also a protease specific for Asp residues [24]. Moreover, the unusual specificity for an Asp-containing cleavage site is shared with one of the cytotoxic T cell granule proteins, granzyme B. Although ICE, fragmentin 2 and granzyme B share no primary sequence homology, their similar action in initiating apoptosis suggests that they may act on a common intracellular target whose proteolytic cleavage is a key event in triggering apoptosis. Thus, it appears that the immune system has 'appropriated' the highly-conserved cell death pathway present in metazoan cells to allow its effector cells to kill infected target cells [25].

Integrated control of cell growth and death in mammals

Apoptosis in mammalian cells and tissues is subject to a variety of regulatory mechanisms that probably have no direct counterparts in the nematode. This may reflect the greater complexity and different structural organization

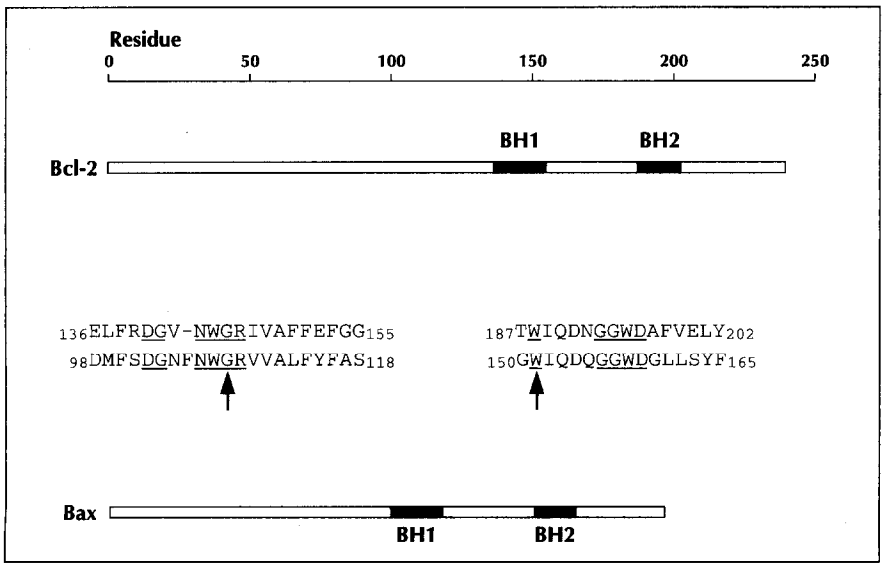

Fig. 1. Key conserved dimerization domains of Bcl-2 and Bax. Evolutionarily conserved residues in each Bcl-2 homology box are shown underlined. Key residues (Gly145 in Bcl-2 BH1, Trp188 in Bcl-2 BH2) involved in dimerization are marked with arrows. Numbering refers to the residue numbers in the human proteins.

of mammals. For example, unlike the case in the worm, substantial proliferation of component cells continues throughout mammalian life, raising the risk of uncontrolled cell proliferation and consequent neoplasia. Moreover, both the nervous and immune systems of mammals are immensely complex structures that arise through an extensive process of iterative matching between cells. In the nervous system, matching involves the forging of appropriate synaptic connections, which are necessary to establish functional neural networks. Excess cells that fail to connect appropriately with target cells are eliminated by apoptosis. In the immune system, autoreactive cells share a similar apoptotic fate.

Recent evidence indicates that much of the machinery controlling mammalian cell proliferation is intimately connected with the control of apoptosis and that this acts as an important safeguard against cancer [13, 26]. Thus, many dominant growth-promoting oncogenes are also potent triggers of apoptosis [13], a fact most graphically demonstrated in the case of the proto-oncogene c-*myc* Expression of c-*myc* is necessary, and in many cases appears sufficient, for cell proliferation (reviewed in [26]). Deregulated c-*myc* expression is virtually ubiquitous in all tumour cells [27] and is associated with inability to withdraw from the cell cycle [28–30] and suppression of differentiation [31–35] .

Paradoxically, c-*myc* is not only a potent inducer of cell proliferation but also a powerful trigger of cell death. Indeed, substantial oncogenic synergy is apparent between c-*myc* and the anti-apoptotic gene *bcl*-2 [36–38] implying that cell death is an important limitation in the oncogenic progression of proto-tumours with activated c-*myc*. Recent *in vitro* studies of the induction of cell death by c-Myc have demonstrated that death occurs by the active process of apoptosis and is aggravated by deprivation of specific growth factors or application of cytotoxic agents [29,39]. Moreover, identical regions of the c-Myc protein are required for both growth promotion and induction of apoptosis. These include the amino-terminal *trans*-activation domain of c-Myc and the carboxy-terminal DNA-binding and dimerization bHLH-LZ domain [29] mediating dimerization with the Max protein [30]. These regions of the c-Myc protein are also probably important in c-Myc's role as a sequence-specific DNA binding transcription factor, implying that c-Myc induces apoptosis via a transcriptional mechanism — presumably by modulating appropriate target genes that regulate cell viability.

The notion that c-Myc, a ubiquitous and essential mediator of cell proliferation, is also a potent inducer of programmed cell death raises the paradox of how proliferating cells, in which c-Myc must be active, manage to avoid setting off the apoptotic pathway that c-Myc initiates. The solution to this conundrum appears to be that cell viability is regulated by specific signal transduction pathways within cells that are activated by discrete cytokines that function as survival factors [5,40]. Proliferating somatic cells *in vivo* survive only so long as they are receiving the correct survival factors. As a substantial level of apoptosis is tolerated in tissues [2], the limited availability of survival cytokines in the somatic milieu therefore acts as a potent homeostatic mechanism to curtail inappropriate cell expansion and neoplasia. Carcinogenic mutations that drive unscheduled cell proliferation will be lethal as soon as the affected cell and its progeny outgrow the paracrine supply of available survival factors [13,26,29]. In effect, surveillance against neoplasia is 'hardwired' into the proliferative machinery of mammalian cells.

Targets for intervention in apoptotic pathways

Modulation of dimerization

The interaction between Bcl-2 and Bax is a prototype for a major mechanism regulating apoptosis. Bcl-2 and Bax each homodimerize and heterodimerize with each other. Recent evidence favours the notion that Bcl-2–Bax heterodimers promote survival, while Bax homodimers may directly activate apoptosis. Thus, relative intracellular levels of Bcl-2 and Bax act as a rheostat which determines the innate tendency of a cell to survive or undergo

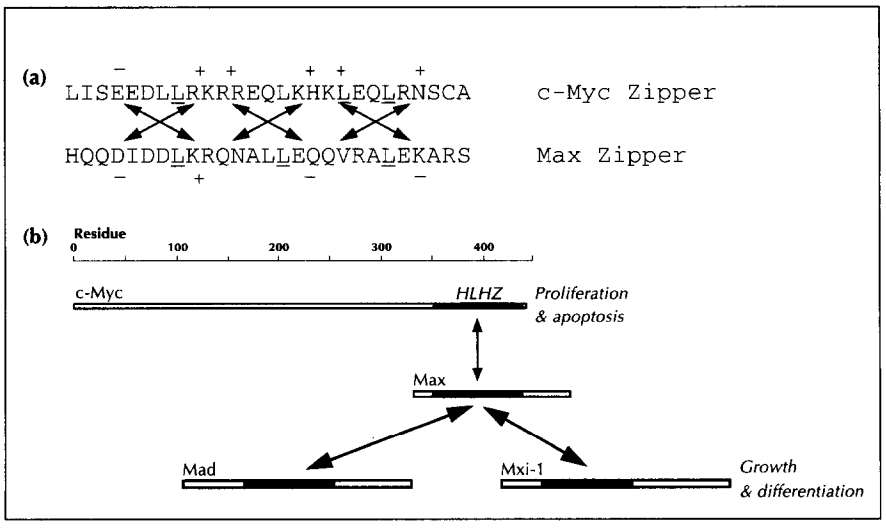

Fig. 2. Interactions among the members of the Myc–Max family. **(a)** Interactions between residues of opposite charge responsible for specificity of interaction between leucine zippers of c-Myc and Max (after [43]). Underlined Leu residues form the leucine zipper. Positive and negative charges are shown. **(b)** The Myc–Max–Mad–Mxi-2 network. The HLHZ dimerization domains of each protein are shaded. Max is central to the network, interacting with all three other proteins. Max is required for DNA binding and function of both c-Myc (promoting cell proliferation and apoptosis) and Mad/Mxi-1 which oppose the effects of Myc and promote differentiation. Max is, thus, an obligate component of both opposing functions in the network.

apoptosis [15]. Other members of the Bcl-2 family also interact with each other and form an extensive network of positive and negative regulators of cell viability. The dimerization interfaces between Bcl-2 and Bax therefore represent an intriguing target for the pharmacological modulation of cell viability. Two domains within Bcl-2 that are highly conserved in evolution, the Bcl-2 homology (BH) domains BH1 and BH2, are both essential for heterodimerization with Bax [41]. Site-directed mutagenesis has identified key residues involved in dimerization (Fig. 1): specifically, the highly conserved Gly145 in BH1 and the equally conserved Trp188 in BH2 [41]. At present, there are no clues as to the structural basis for dimerization via the BH1 and BH2 domains or why the residues known to be critical are so important. Nonetheless, the identification of comparatively small molecular surfaces such as these that are crucial for biological function provides the basis for both rational and empirical drug development.

Interactions between the growth and apoptosis-promoting polypeptide c-Myc and its partner Max have also been extensively defined [42]. Both c-Myc and Max possess extended amphipathic α-helical helix-loop-helix zipper (HLHZ) domains. The hydrophobic surfaces of each HLHZ domain interact to mediate dimerization, while specificity of the interaction is dictated by interactions between key residues of opposite charge on each domain ([30,43]; Fig. 2a). Max is involved in further interactions with yet other HLHZ proteins, Mad [44] and Mxi-1 [45], whose actions seem to be to suppress c-Myc functions. c-Myc, Max, Mad and Mxi-1 together form an extended network of interactive polypeptides [42] (Fig. 2b) that are important regulators of cell proliferation, differentiation and viability. The ability to interfere selectively with the interactions between proteins in this network seems certain to be useful. But it is difficult to predict the precise biological consequences of interfering with such a complex iterative network. One potential problem with development of agents that interfere with Myc–Max–Mad HLHZ interactions is the extended surface over which HLHZ domains interact. This may mean that it is not possible to block the interaction with low-molecular-weight inhibitors. Nonetheless, construction of α-helical peptides with dominant interfering activity peculiar to each dimerization pair should be possible and such reagents may be useful if effective systems can be designed to facilitate their specific delivery into cells.

ICE proteins — proteolytic triggers of cell death

As outlined above, the ICE-type proteases are the current best candidate for actual triggers of apoptosis in mammalian cells. Active human ICE is composed of two subunits (p20 and p10) that are cleaved from a single proenzyme (proICE); both subunits are required for enzymatic activity *in vitro* [46]. ICE is a very specific protease, first identified by its ability to activate pro-Interleukin-1β by cleavage at two critical Asp residues. Moreover, ICE is autocatalytic at high concentrations (active ICE cleaves the proICE to generate more active enzyme), an excellent attribute for the trigger of an irreversible cascade.

It now seems unlikely that ICE itself is the actual apoptotic trigger in cells. Instead, it appears that different members of the family are important in the apoptosis of various cell lineages. The *nedd2* gene encodes a small protein with 27 % identity to the p10 subunit of ICE and the carboxyl-terminus of the nematode *ced-3* death gene. Its expression is implicated in apoptosis in neurons and kidney during development [17]. Another ICE homologue, ICH, is very widely distributed in tissues and is expressed as two splice variants. The longer form, ICH_L, triggers apoptosis in various cell types, while the shorter form, ICH_S, appears to suppress apoptosis, possibly by interfering with ICH_L [18]. Yet another homologue of ICE, prICE, was identified by Earnshaw and colleagues [19] in an elegant *in vitro* model system that mimics aspects of nuclear fragmentation during apoptosis. This cysteine protease activity has specificity for poly-ADP ribose phosphorylase (PARP); it shares overlapping substrate specificity with ICE, cleaving PARP at the sequence EVD/G [19], and is essential for nuclear condensation and fragmentation in the system used by these authors.

Protease triggers of apoptosis make obvious and attractive potential targets for modulation by drugs. It is relatively straightforward to design effective peptide-based inhibitors on the basis of the known sequence specificity of each protease. Indeed, the prICE substrate derivative YVAD-chloromethyl ketone has been shown to be an effective inhibitor of prICE-induced nuclear condensation and the YVAD peptide can be used to isolate prICE protein. The recent elucidation of the structure of ICE [23] is certain to facilitate rational drug design of inhibitors of these important triggers of apoptosis.

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