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News and Commentary

Apoptosis Timeline

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This timeline of cell death (Figure 1), illustrates how independent strands of research coalesced in the field known as apoptosis – currently the hottest field of biological research. Although the fact that cells die during normal development was recognized over 150 years ago,[1] this was forgotten, only to be re-discovered several times until the influential review by Glucksmann in 1951.[2] Even after this time, up until the late 1980's, study of physiological cell death processes, in which an organism's cells activate intrinsic mechanisms for the purpose of killing themselves, remained relatively obscure, usually with less than 10 papers published each year.

Initially, analysis of cell death was mainly morphological, and between the late 1800's and 1960's elegant figures were published illustrating the light (see reviews by Clarke and Clarke and Lockshin)[82,83] and electron microscopic[3] features of cell death, such as cell shrinkage, chromatin condensation, break-up of the cell and its engulfment.

Even well after the proposal of the term 'apoptosis' for cell death in 1972,[8] interest remained low. The 'modern' era of cell death research, and the explosion of interest in the field, came with the identification of the biochemical and genetic processes that implement it, beginning with recognition of the first component of the cell death system, Bcl-2, in 1988.[20] Since then, growth of the field has been exponential, and currently over 200 publications appear every week that refer to 'apoptosis'. A genetic understanding of cell death has primarily come from study of *C. elegans*, in which 131 of the 1090 somatic cells formed in the hermaphrodite are fated to die during development.[16] This started with the recognition of cell death in the worm in 1976,[11] and generation of the first *ced* (*ce*ll *d*eath abnormal) mutants in 1983.[14] In 1982, in a journal that unfortunately folded soon after, a paper appeared providing evidence that cell deaths in the worm were caused by a process that was specific for cell death, and had no other role, indicating that cell death in the worm is an active process whose only purpose is to remove unwanted cells.[13] Similar conclusions were reached earlier in vertebrate systems, such as when Tata showed that cell deaths during tadpole metamorphosis could be inhibited by cycloheximide, and therefore required the cell's own proteins.[6]

At this time, the term most commonly used for the study of these cell death was 'programmed cell death', first used in 1965 to describe developmental cell deaths in insect systems by Lockshin and Williams.[5] The term 'apoptosis' was proposed in 1972 by Kerr and colleagues,[8] who realized that the morphology of cells dying due to toxins or hormones resembled that described for developmental cell death by Glucksmann.[2] For Kerr, this did not mark the beginning of apoptosis research, because he had been studying it continuously since his first publication on cell death in 1965;[4] rather, it marked the adoption of a new terminology, because until then he had used the terms such as 'shrinkage necrosis'.

The first marker of physiological cell death that did not rely on morphology came with the recognition that cell death is usually accompanied by rapid activation of endonucleases.[10] Subsequently, 'ladders' seen after electrophoresis of cleaved DNA[9] were specifically associated with apoptosis.[12] It took a further 17 years to identify the major endonuclease responsible (DFF/CAD).[63,64] The observation that phosphatidyl serine is exposed on dying cells[32] provided another convenient marker of apoptosis, and also gave an early lead into how dead cells are recognized prior to their engulfment. Although genetic analysis of cell death progressed most rapidly in the worm, with identification of more and more *ced* mutant lines,[16,29] biochemical analysis of cell death was faster in mammals. While Bcl-2 was cloned in 1986,[17,18] and its role in cell death was established in 1988,[20] the first *ced* gene to be cloned and sequences was *ced-4* in 1992.[31]

Comparisons of the morphological and anatomical features of developmental cell deaths in invertebrates and vertebrates have been made since 1969,[7] but unification of the molecular processes of cell death did not occur until 1992, when it was shown that the human *bcl-2* gene could inhibit developmental cell death in the worm.[30] This united 'apoptosis' in vertebrates with 'programmed cell death' in invertebrates, showing them to be the same, evolutionarily conserved process, and it meant that discoveries based on genetics in *C. elegans* could be applied to analysis of apoptosis in mammalian cells.

While Bcl-2 was the first component of the apoptosis mechanism to be recognized, it had been cloned not because it was a cell death gene, but because it is translocated in follicular lymphoma, one of the most common cancers of blood cells in humans. Initially, it was assumed that *bcl-2* may be like other oncogenes involved in translocations, such as *abl* and c-*myc*, and be a promoter of cell proliferation, but it turned out that when *bcl-2* was over-expressed, it did not stimulate cell division, but prevented cells from dying when growth factor was removed.[20] These experiments therefore not only identified Bcl-2 as a component of the apoptosis mechanism, but showed that inhibition of cell death could ultimately lead to

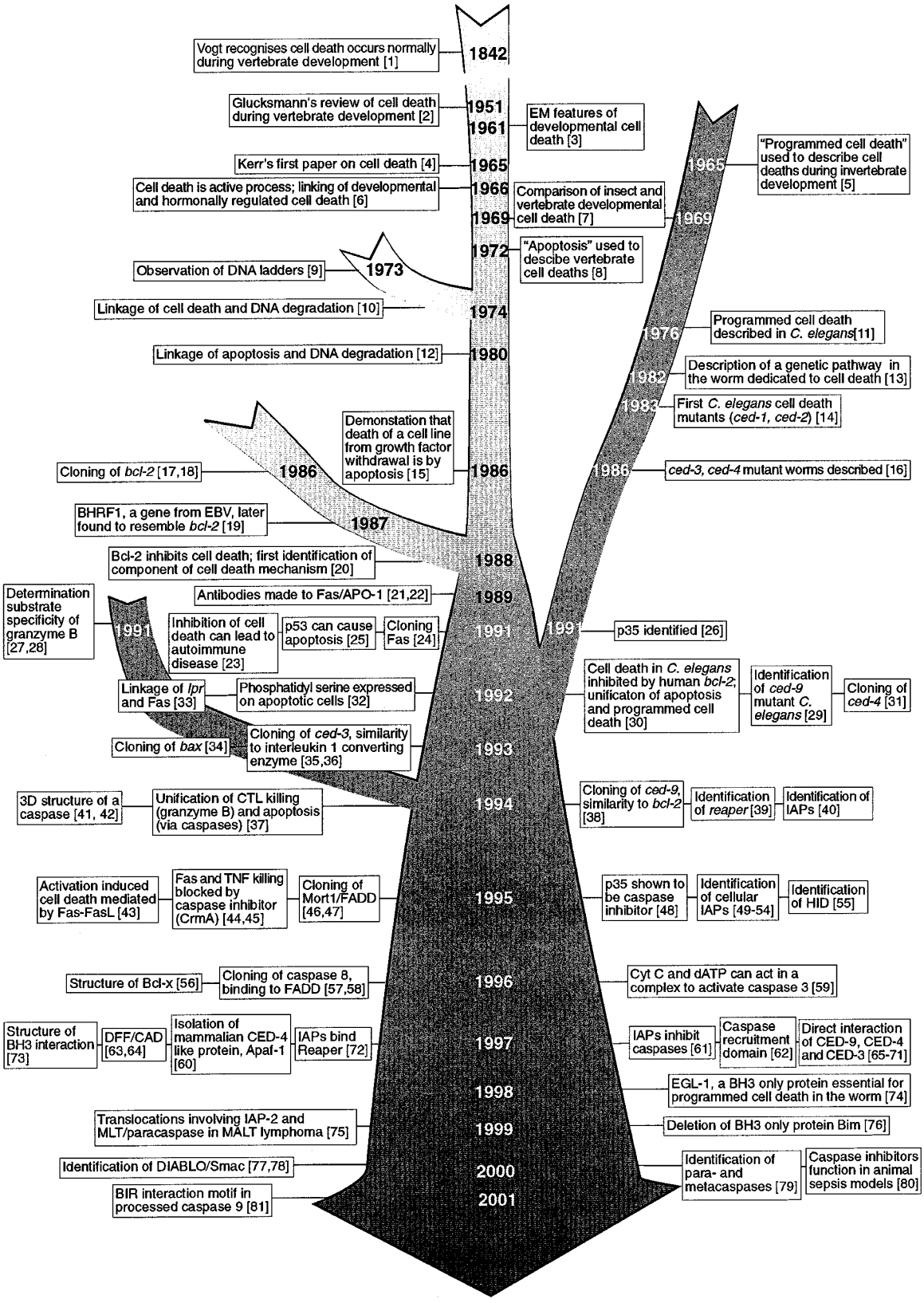

Figure 1 Over 50 000 papers have been published on apoptosis, and it is only possible to give an indication of some of them in this figure. Many, many, important papers have therefore been omitted. In most cases, only the first member of a protein family is mentioned (that is why the cloning of all the Bcl-2 family members, or all the caspases, are not listed). This figure only depicts findings that are widely accepted. There are many important molecules, or findings, whose roles or interpretations are recent or remain controversial, and have therefore not been shown (e.g. ceramide; the channel forming role of Bcl-2 family members; mitochondrial permeability transition; DAP kinase; DAP3, Survivin; DAXX; FAP1; reactive oxygen intermediaries; BAG-1; AIF; AVEN, etc., etc.). Other (non-caspase mediated) mechanisms of cell death, and cell death research on non-metazoans, has not been included

cancer in humans. The realization that one of the roles of p53, the most commonly mutated gene in human cancers, is to cause apoptosis,[25] further emphasized this link, as did the demonstration that p53 causes apoptosis via the mechanism that can be blocked by Bcl-2.[84] Bcl-2 also provided the first experimental evidence linking inhibition of cell death with autoimmune disease, when it turned out that on certain genetic backgrounds transgenic mice expressing *bcl-2* in their lymphocytes developed a disease resembling systemic lupus erythematosus.[23] This link was further strengthened when the gene altered in *lpr* mice, which also develop a lupus-like autoimmune syndrome, turned out to be CD95 (Fas/APO-1),[33] a TNF receptor family member[24] that was known to signal apoptosis when crosslinked by antibodies.[21,22] Furthermore, mice lacking *bim*, which encodes a so-called 'BH3 only' pro-apoptotic Bcl-2 homologue, also develop autoimmune disease.[76]

The effector proteases of apoptosis, now known as caspases, were first recognized when the *ced-3* gene, which is essential for programmed cell death in the worm,[16] was cloned and sequenced,[35,36] and found to resemble the mammalian gene for the cysteine protease interleukin 1β converting enzyme, which had been cloned in 1992.[85,86] Crystallography revealed that active caspases are hetero-tetramers formed from inactive zymogens.[41,42] This focussed interest on what activates caspases, and what inhibits them.

Key findings have included the elucidation of a caspase activation pathway that originates in the plasma membrane, and proceeds from CD95, via the adaptor FADD, to activate caspase 8,[44,45,57,58] and the findings that in *C. elegans* the adaptor CED-4 directly binds to and activates the caspase CED-3.[67,68,70,71] Identification of mammalian homologues of these proteins (Apaf-1 and caspase 9)[60,87] showed that a similar pathway operates in mammals, and revealed cytochrome *c* to be a molecule capable of activating Apaf-1.[59] Many of the interactions between these cell death molecules involve related protein-protein interaction motifs termed death domains, death effector domains and caspase recruitment domains.[62]

While it is clear that anti-apoptotic Bcl-2 like proteins act upstream of caspases to prevent their activation, and pro-apoptotic Bcl-2 family members such as Bax[34] promote caspase activity, debate remains about exactly how they work. Biochemical experiments using *C. elegans* proteins have suggested that CED-9 (the worm homologue of Bcl-2) inhibits cell death by directly binding to CED-4,[65–69] but it is unclear whether similar direct interactions occur between their mammalian counterparts.

Solving the structure of Bcl-x,[56] a Bcl-2 family member, raised the alternative possibility that these proteins act as membrane pores or ion channels, to somehow influence release of pro-apoptotic molecules such as cytochrome *c* from the mitochondria. From both structural studies, and genetics in *C. elegans*, it is, however, clear that anti-apoptotic Bcl-2 family members can be bound, and antagonized by, 'BH3 only' proteins such as Bim and Noxa in mammals,[76,88] and EGL-1 in the worm,[74] thus increasing the likelihood that a cell will undergo apoptosis. BH3 only proteins are key determinants of cell death in worms and vertebrates. All somatic developmental cell death in *C. elegans* require EGL-1,[74] and in mammals p53-dependent apoptosis seems to be signalled in large part via Noxa.[88] The discovery that the helical BH3 domain of one Bcl-2 family member can bind to a hydrophobic pocket on the surface of another[73] has helped explain how pro-death Bcl-2 family proteins antagonize their anti-apoptotic cousins.

Not all physiological cell deaths in animals are cell autonomous (i.e. cell 'suicide'), sometimes one cell kills another cell (i.e. cell 'murder'). In *C. elegans*, death of the male linker cell is non-cell autonomous,[16] and in mammals, cytotoxic T cells (CTL) kill other host cells, especially those infected by viruses. Targets of CTL killing display the characteristic features of apoptosis,[89] and it became clear why when the mechanisms involved in CTL killing were elucidated. CTL can kill by perforin-dependent, granule exocytosis, which involves granzyme B, a serine protease with a similar substrate specificity to the caspases,[27,28] or via CD95L-CD95 interactions, which activate caspase 8.[57,58] Knowledge of the enzymes involved in CTL killing therefore allowed unification of cell autonomous and non-cell autonomous cell deaths, and explained the shared apoptotic appearances.[37]

CTL killing illustrates the role of apoptosis in defense against viruses. But viruses have been selected that carry inhibitors of apoptosis. Several direct inhibitors of caspase activity were first found in viruses, and for some, cellular homologues were later identified. The first caspase inhibitor found was CrmA, a product of cowpox virus that was known to inhibit interleukin 1β converting enzyme (caspase 1),[90] but is now known to also inhibit caspase 8, and thereby can block CD95 and TNFR signalled apoptosis.[91] The gene for p35 was first found in baculoviruses,[26] as were the first inhibitor of apoptosis (IAP) genes.[40] Both p35 and IAPs act by binding directly to, and thereby inhibiting, active caspases.[48,61] Several mammalian IAP homologues have been discovered,[49–54] and one, c-IAP2, is commonly translocated in MALT lymphomas, where it is expressed as a fusion with the MLT/paracaspase gene product.[75,79]

In insects three different proteins, Reaper, HID and Grim,[39,55,92] promote apoptosis by antagonizing the IAPs,[72] and a mammalian protein, Smac/Diablo, has been found that inhibits mammalian IAPs in a similar way.[77,78] The identification of a similar BIR-interacting N-terminal motif in processed caspase 9 revealed how Smac/Diablo can displace caspase 9 from IAPs.[81] A tremendous effort is now being expended to discover even more about how apoptosis works, and to resolve some of the controversies that remain. It is still not clear how Bcl-2 family members work, or how cytokines prevent default activation of the cell death mechanisms, or even whether in mammalian cells prevention of caspase activity will allow long-term survival. The answers to such questions are not trivial, but will determine to what extent these wonderful, yet curiously delayed, discoveries in basic science will be easily applied to the development of novel therapeutic agents for the treatment of diseases in which cell death fails to occur or occurs inappropriately. The first non-peptide caspase inhibitory drugs are proving useful in animal models of sepsis,[80] suggesting apoptosis-based therapies are not far away.

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