Programmed cell death

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Programmed cell death (PCD) occurs during development in all the animals studied so far, but the molecular basis has been recently discovered. Apoptosis is a highly organized and genetically controlled mechanism which helps in maintaining the homeostasis in multicellular organisms. This paper reviews the recent developments in the field of PCD. Emphasis has been laid on the recent developments in the Caenorhabditis elegans cell death programme. This process is well characterized by some biochemical and cytological events. Three key genes in the PCD of C. elegans have been characterized. The ced-3 and ced-4 genes function in the killing of cells while the ced-9 gene prevents this phenomenon. The ced-9 (cell death suppressor) and ced-3 (cell death inducer) genes encode proteins which share a functional and structural homology with the mammalian proto-oncogene bcl-2 and interleukin-1β converting enzyme, respectively. These findings reveal key molecules that control life and death decisions in vertebrates. Characterization of these genes has revealed that the process of PCD is evolutionarily conserved and has shed light on the molecular nature of the apoptotic machinery.

It has been known since the 1950s that many cells die naturally during development but these ‘killer’ molecules were identified only recently. This review will focus on the recent findings that have revealed the critical players in this field of programmed cell death (PCD). It is a suicide mechanism, which plays an indispensible role in the development and maintenance of adult tissue homeostasis. This mechanism is genetically encoded and conserved throughout, from the unicellular to the multicellular organism in both plants and animals. The term ‘apoptosis’ usually refers to a morphological type often observed in PCD. This phenomenon involves cytoplasmic and nuclear condensation and fragmentation and is associated with RNA and protein syntheses. In most of the model systems studied so far PCD follows an apoptotic pattern, however other morphological types have also been shown. Lockshin and Williams emphasized that deaths are somehow programmed into the developmental plan of an organism and this could possibly be the reason for having predictable cell deaths at predictable places and times during development.

In recent years evidence has come up suggesting prevention of cell death by signals from other cells. In their landmark paper, Kerr, Wyllie and Currie proposed an active intracellular death programme that could be activated or inhibited by a variety of physiological stimuli. It was only in the 1980s that this suicide programme was accepted after the genetic studies carried in Caenorhabditis elegans by Horvitz and his group. They have been successful in identifying the genes responsible for the death programme and its control. Soon after, homologues of these genes controlling cell death in mammals, yeast, plants, etc. were identified. The molecular characterization of all the known cell death genes from different systems indicates the conservation of the cell death programme through evolution.

Is PCD needed during development?

Cell suicide provides an efficient mechanism for eliminating unwanted cells. The question arises; What are these unwanted cells?

(a) They could be cells like the vertebrate neurons which are produced in excess. Mice in which CPP32 (caspase-3) has been deleted by targeted gene disruption die because of excess of cells in their central nervous system. In other words, reduced apoptosis in the brain brings about premature lethality in CPP32 deficient mice.

(b) Those cells, which have already undergone their function and are no longer needed, undergo death. For example, a tadpole tail during metamorphosis. During mouse embryonic development generation of oxidative stress is a common requirement for cells undergoing the death programme.

(c) Some cells have to undergo death (or are sacrificed) for sculpturing the body parts. For example, in digit formation of higher vertebrates where the interdigitatory region succumbs to death.

(d) PCD functions to eliminate cells that are abnormal, misplaced, non-functional or potentially dangerous to the organism. One good example is lymphocytes, which fail to produce functional antigen-specific receptors and are thereby rendered useless.

(e) Some cells are needed only by a particular sex and therefore they have to undergo PCD. For example the Mullerian duct is needed in females only while the Wolffian duct is needed in males only. Therefore, the counterpart of the other sex type must undergo cell death for normal development to occur.

Thus there are many other kinds of cells undergoing PCD whose functions are not known. Some cells die even
before they get a chance to function. PCD-deficient flies die early in development\textsuperscript{19} while mutant nematodes for PCD can only survive under laboratory conditions\textsuperscript{20}. Probably nature (via the organism) selects the 'best' cells which would be needed for successful development of the organism.

**Systems for studying apoptosis**

Apoptosis occurs in two physiological stages following an intrinsic or extrinsic signal to the cell. The cell enters the 'condemned' or committed phase in a cell autonomous fashion and finally arrives at the 'execution' phase. The condemned phase could vary with time. Apoptosis can be induced in cells under *in vitro* conditions by a number of ways. One of the classical systems is exposure of thymocytes to glucocorticoids. Other methodologies include DNA damage either by irradiation, exposure to drugs that inhibit topoisomerase, withdrawal of growth factors from growth media, cell cycle perturbation, exposure to inhibitors/activators of kinases or phosphatases, interference with Ca\textsuperscript{2+} homeostasis, overexpression of p53, members of Ced-3/ICE and many more.

There is probably more than one physiological pathway through the condemned phase and due to its variability in time lengths, it has been difficult to elucidate the execution phase pathway. The following are a few successful strategies developed for the analysis of apoptotic execution:

(a) To develop a cell-free system involving isolation of normal and preapoptotic nuclei and exposing them to buffers of different ionic strengths\textsuperscript{21,22}. This approach made available the data on the nucleases involved in DNA fragmentation.

(b) To develop a cell-free extract from condemned apoptotic cells and mix them with nuclei to monitor the course of apoptosis\textsuperscript{23}. Studies indicated the dominance of cytoplasm over the nucleus in the execution of apoptosis.

(c) To develop cytoplasmic extracts prepared from the synchronized S/M phase of condemned cells and induce isolated nuclei to undergo apoptotic execution\textsuperscript{24}.

**Morphological changes during apoptosis**

Studies indicate that although apoptosis is an asynchronous process it can be defined in terms of reproducible morphological changes that can be used to place other events, for example, commitment to die, in a temporal sequence\textsuperscript{25,26}. Initiation of PCD results in the shrinking of cytoplasm and condensation of nuclear chromatin\textsuperscript{26,28}. Cytoplasmic changes include clustering of organelles and formation of arrays of ribosomes. Vacuoles derived from the endoplasmic reticulum produce a 'blebbing' of the cytoplasm\textsuperscript{27,28}. The entire nucleus in some cases condenses into a single dense ball whereas in others the chromatin gets fragmented and surrounded by a nuclear envelope called 'apoptotic bodies'\textsuperscript{26,28}. Changes in chromatin are accompanied by changes in the nuclear envelope. The nuclear pores redistribute by sliding away from the surface of the condensed chromatin domains and accumulate between them\textsuperscript{29,30}. This movement of the pores suggests that association with a nuclear lamina no longer restricts them. In fact, the lamina disassembles during apoptosis\textsuperscript{30,31}. Lamina disassembly is driven by phosphorylation of the lamina subunits during mitosis whereas in apoptosis disassembly is accompanied by proteolysis\textsuperscript{31,32}. Cleavage of selective protein subsets is a key event in the execution of apoptosis. Protein degradation may play a role in the structural alteration that may result in cell self-destruction, but it may also function as a switch in the decisions between apoptosis and cell proliferation\textsuperscript{33}. Investigations using high resolution 2D gel electrophoresis combined with computer-assisted image analysis indicates the potential involvement of nuclear matrix proteins in the apoptotic process\textsuperscript{34}. The biochemical feature most commonly associated with apoptosis is the presence of 'fragmentation nuclease' and/or 'domain nuclease'. Domain nuclease cleaves the genome into 200–300 and 30–50 kb pieces\textsuperscript{35}, although there is no evidence to indicate their precise sites involved in cleavage. The nuclease requires different divalent cations and is not inhibited by Zn\textsuperscript{2+} or inhibitors of serine protease\textsuperscript{36}. Removal of Zn\textsuperscript{2+} ions does not increase the percentage of cells undergoing H\textsubscript{2}O\textsubscript{2}-induced cell death but rather results in rescue from death\textsuperscript{37}. No convincing candidate has been identified yet, though evidences point towards topoisomerase kind of activity\textsuperscript{38} which also has been confirmed by the use of its inhibitor etoposide in many systems. The fragmentation nuclease is active in the second phase resulting in an oligonucleosomal 'ladder' pattern\textsuperscript{39} and has been identified as DNAase I (ref. 40) and DNAase II (ref. 41) and a protein related to cyclophilin A (ref. 42). The mode of action and requirements for DNAase I and II are very specific. The DNA breaks produced in apoptosis have a 3' OH group. Apoptotic cells appear to undergo acidification that could permit action of DNAase II (ref. 43). Till date no clear-cut evidence is available regarding the identification of apoptotic nuclease. Different cells probably utilize different nucleases. Using isolated thymocytes, Sun and Cohen\textsuperscript{44} have demonstrated that fragmentation of large fragments (200–300 kbp) is Mg\textsuperscript{2+} and not Ca\textsuperscript{2+} dependent, while the cleavage to 50 kbp fragments and not the oligonucleosomal cleavage is Mg\textsuperscript{2+} dependent but is facilitated by Ca\textsuperscript{2+}. Recent studies have shown that DNA fragmentation associated with apoptosis is mediated by a DNA fragmentation factor (DFF)\textsuperscript{45} which is activated by caspases, mainly caspase-3 (refs 46–50). DFF is composed of 2 protein subunits: a 40 kD caspase activated nuclease (DFF40/CAD) and its 45 kD inhibitor (DFF45/CAD)\textsuperscript{46–50}. Cleavage of DFF45/CAD by caspase-3 releases DFF40/
CAD from its inhibitor leading to induction of nuclease activity, nuclear condensation and DNA fragmentation in vitro. However, the molecular basis for DFF40/CAD function and its regulation by DFF45/ICAD is poorly understood.

Based on the amino acid homology to the N-terminal region of DFF45/ICAD, a conserved family of proteins named CIDEs were identified. CIDEs are proapoptotic proteins and induce DNA fragmentation, membrane blebbing and nuclear condensation. This activity is inhibited by DFF45/ICAD. Mutational analysis revealed that the CIDE contains 2 domains (N and C). CIDE-C is necessary and sufficient for apoptosis whereas CIDE-N acts as a regulatory domain required for the inhibitory activity of DFF45/ICAD. Recently it has been shown that DFF40/CAD has two domains with distinct biological functions. The N-terminal region has a conserved domain for regulating the activation of apoptosis.

Internucleosomal DNA cleavage should not be the sole criterion for the identification of apoptosis. If there is no early DNA fragmentation PCD is marked by extensive vacuolization. Apoptotic bodies express surface markers helping them to be rapidly phagocytosed by macrophages or neighbouring cells which digest them. All this takes place without any leakage of cellular constituents. Now it is possible to distinguish between apoptotic and necrotic development in living cells using fluorescent microscopy. This was made possible in neuroblastoma cell line expressing nuclear pore protein tagged with green fluorescent protein.

PCD in invertebrates

*C. elegans*

Among the 1090 somatic cells generated during *C. elegans* hermaphroditic development, 131 die, a phenomenon observed in every individual at specific developmental stages and position. This suggests that PCD is strictly determined in cell lineage during development of the nematode. Dozens of genes and their functions in the death programme have been identified. *ced-3* and *ced-4* genes function in the killing of the cells while *ced-9* prevents this phenomenon. *ced-9* is a negative regulator of *ced-3* and *ced-4* and acts upstream of these genes to prevent them from killing the cells. Cloning and characterization of *ced-3* gene indicated the product to be a member of the ICE family.

The second *C. elegans* gene suppressing cell death is *dad-1* (ref. 59). Overexpression of either nematode or mammalian *DAD-1* prevents PCD in *C. elegans* suggesting that it may be a direct and proximal inhibitor of cell death. Interaction of *dad-1* with other cell death genes is not yet known.

Recent studies suggest that apoptosis eliminates excess germ cells which act as nurse cells to provide cytoplasmic components to maturing oocytes. Germ cell death requires ras/MAPK pathway activation and is suggested to maintain germline homeostasis.

*Drosophila*

Contributions by Stellar and his colleagues have made *Drosophila* a good model system to study the mechanism of PCD. These investigators initiated a genomic screen for chromosomal deficiencies, which under homozygous conditions would prevent embryonic PCD. They revealed the identification of these genes on the same loci, namely *reaper*, *head involution defective* (hid) and *grim*. Isolation and molecular characterization of the *reaper* gene shows a novel 65 amino acid protein. The *reaper* expression pattern in embryos closely resembles the patterns of cell death and overexpression appears to be sufficient to induce apoptosis.

Identification and molecular characterization of the *hid* gene has already been done. *hid* mutants decrease the level of cell death while overexpression promotes apoptosis, which can be suppressed through expression of baculovirus p35. The question which arises is how do *reaper* and *hid* induce cell death as neither of them show significant sequence homology to any of the core death molecules of any known system. It could be possible that they are not a part of this machinery but act as upstream regulators.

The genetics of *C. elegans* and *Drosophila* has an added advantage over the other systems. In these systems additional cell death genes can be easily identified. In fact, they could contribute more towards the understanding of the molecular mechanism of PCD.

PCD in the nematode, *C. elegans*

To date 14 genes of the PCD pathway have been identified. The whole process of PCD is distinctly divided into four steps: (a) Determination of cells to enter PCD; (b) Execution of PCD; (c) Engagement of dead cells; (d) Degradation of dead cells.

The decision of cell death is most likely made through cell lineage, and the genes involved in this decision making can be considered as cell fate determination genes. Once these cells are determined to die, genes responsible for the execution of cell death are activated in them. Two important genes required for PCD are *ced-3* and *ced-4* (ref. 10). If any of the PCD genes are mutated the phenomenon of inhibition is very specific. Sexual maturity attained by *ced-3* and *ced-4* mutants is slowed approximately by 30% in comparison to the wild. This evidence reveals the importance of these genes for increasing energy efficiency.

Genetic mosaic analysis was carried out to find the site of action of *ced-3* and *ced-4* genes which undergo inde-
dependent cell death programme. It is possible to observe PCD as a result of murder or suicide by the cells. In C. elegans, a number of evidences are present which support the suicide mechanism of PCD. Some of them are as follows: (a) Size bias of the cells (smaller cells undergo PCD) suggest predetermination of cell fate; (b) Cells undergoing PCD do not show any differentiated phenotypes, suggesting their failure in competing for the targets; (c) Ablation of engulfing neighbouring cells with laser beam failed to rescue the dying cells; (d) Some of the doomed cells are already embraced by engulfing cells before they are born.

*ced-3* and *ced-4* undergo independent cell death programmes and no cell-to-cell interactions (or signals) are needed for its initiation. Unlike them, the male specific linker cell requires another cell and dies much later during development – this could possibly be a case of murder rather than suicide.

Molecular analysis of *ced-3* and *ced-4* showed dose-dependent phenotypes. All mutations of *ced-4* eliminated the expression of *ced-4* mRNA whereas the *ced-3* mutations were of missense kind. The *ced-3* gene, which is maximally expressed during embryogenesis when PCD is very active, encodes a 2.8 kb mRNA. Since at any given time only 2–3 cells die, it may not be restricted to dying cells only. Analysis of the sequence reveals that it codes for a 503 amino acid protein. It is hydrophilic and does not contain any potential signal peptide transmembrane domain. A less conserved region (amino acid residues 107–205) found to be rich in serine is found in related nematode species but no mutation in this region has been identified. The amino terminal region is well conserved and mutation analysis reveals that the carboxy terminals actually regulate the function of the protein. The non-serine rich region shares a homology with human and murine interleukin-1β converting enzymes (ICE)63. IL-1β is synthesized as an inactive 33 kD polypeptide, which must be processed by proteolytic cleavage in order to be secreted. ICE, the cysteine protease is responsible for the IL-1β maturation whereby it cleaves at the carboxy terminal side of the obligatory aspartate residue to release a 153 amino acid polypeptide. CED-3 protein and human ICE protein share a 29% homology mainly in the carboxy terminal region. A stretch of 115 residues (amino acid residues 249–360) is 43% identical between CED-3 and human ICE and contains a pentapeptide QACRG (361–365 positions) surrounding a cysteine important for the functioning of ICE.

A 2.2 kb mRNA is transcribed from *ced-4* and encodes a 549 amino acid open reading frame and is expressed maximally at embryogenesis and contains two EF hand motifs or calcium binding domains.

*ced-9* is involved in the tight regulation of *ced-3* and *ced-4* genes. The gene product of *ced-9* negatively controls the switch of cell death machinery. PCD is prevented by a gain of function mutation in *ced-9* while loss of function mutation in them causes ectopic cell deaths resulting in embryonic lethality. Analysis of the gene structure and the transcript of *ced-9* suggest that it be in a polycistronic locus. This locus also contains a gene similar to bovine cyt b 560, an inner mitochondrial protein. *ced-9* encodes a 1.3 kb mRNA and is maximally expressed during embryogenesis. It encodes for a 280 amino acid protein having 23% homology with the human Bcl-2 proto-oncogene product. Both *ced-9* and *bcl-2* genes protect cells from death. All these data point to the fact that the PCD pathway involving *ced-9/bcl-2* is a negative regulator of *ced-3/ICE* and is conserved through evolution.

The dead cells are quickly engulfed/phagocytosed and degraded. Six genes (*ced-1, 2, 5, 6, 7, 10*) are involved in phagocytosis of dead cells while *nuc-1* (nuclease deficient) gene is involved in the degradation of pyknotic DNA of dead cells. The function of *nuc-1* gene in PCD is still unclear.

Mammalian homologues of PCD genes from C. elegans

*bcl-2* gene was first identified at the site of translocations common to many human follicular lymphomas, which resulted in overexpression of the gene. This oncogenic ability attributed *bcl-2* as a repressor of PCD. *bcl-2* is a functional homologue of *ced-9* and it can inhibit cell death induced by c-myc68, p53 (ref. 69), TNE70, Fas antigen71 and various chemotherapeutic drugs72.

Overexpression of *bcl-2* does not suppress all types of physiological deaths: in fact data suggest two distinct mechanisms of PCD in vertebrates: one *bcl-2* independent and the other *bcl-2* dependent. *bcl-2* is widely expressed in embryonic tissues including the nervous system, placental trophoblast and interdigital tissue of limb buds73. The expression of *bcl-2* is detected in many different tissues during different stages suggesting its other functions in addition to preventing PCD. *Bcl-2* may be a multifunctional protein whose activity is regulated at the level of transcription, post-translational and protein–protein interactions. Bcl-2 is an intracellular membrane-bound protein associated with inner and outer mitochondrial membranes, nuclear envelope and endoplasmic reticulum. One of the most common features of these compartments is the production of peroxides. There is a stretch of 19 hydrophobic amino acids at the carboxy terminus, which is necessary for *bcl-2* to prevent PCD. Reduction in mitochondrial membrane potential (ΔΨm) and generation of reactive oxygen species are early events in apoptosis. Cell death is associated with decrease in mitochondrial potential relative to mitochondrial mass, suggesting that an accumulation of damaged or dysfunctional mitochondria has occurred.

*Bcl-2* exhibits two main biochemical properties: (i) it modulates intracellular free calcium levels; (ii) it acts in
an antioxidant metabolite pathway and increases the levels of oxygen free radicals which induce lesions in DNA, protein and lipids.

The Bcl-2 family in mammalian systems includes Bcl-2, Bcl-x, McI-1, A1, Bw, Bax, Bak, Bad and Nbk/Bik, in viral systems it includes ASFV, LMW-5, EBV, BHFR-1, HVS ORF 16, in adenoviral systems EB-19k, and in invertebrates it includes CED-9 gene product. Initiation of apoptosis often transpires in the presence of agents that regulate cell survival. Studies on effects of stress-induced ceramide on the anti-apoptotic activity of phosphoinositide-3-kinase pathway show down-regulation of PI (3) K by ceramide results in the inhibition of kinase (Akt) and decreased phosphorylation of the death effector.

Bax was identified as a 21-kDa protein and shares 21.2% homology with Bcl-2 at the protein level. They can form both homo- and heterodimers with Bcl-2. It has been proposed that the ratio of Bcl-2 and Bax is critical in determining whether a cell will enter apoptosis or not. Bcl-2 exerts its actions mainly through heterodimerizations with Bax. In such a competitive binding model, additional proteins that compete for binding to either Bax or Bcl-2 could influence the outcome after an apoptotic stimulus.

The bcl-x gene is expressed in a wide variety of tissues and its mRNA is spliced into two distinct species. The longer mRNA, bcl-xL encodes a protein with 56% homology with bcl-2 and the shorter bcl-xS encodes a protein, which is an internal 63 amino acids deleted version of bcl-xL and bcl-xL have opposite functions. Overexpression of bcl-xS inhibits the ability of bcl-2 to prevent cell death. The first direct evidence that bcl-x is altered in cancer came recently. Tumours harbouring bcl-x insertions had altered bcl-x mRNAs, expressed elevated levels of Bcl-xL proteins and lacked the requirements for cytokines normally essential for cell survival. Therefore, Bcl-xL functions as a key cytokine and regulated anti-apoptotic protein in myelopoiesis contributes to leukemia cell survival. The dimerization domain shares the greatest homology between family members. Two important domains are BHI (residues 138-154 of bcl-2) and BH2 domain (residues 180-196). BH3 is a 28 amino acid region distinct from both BHI and BH2 from Bak or Bax and is sufficient for binding to Bcl-2 or Bcl-xL (refs 85, 86). BH3 is an important component of inducers of apoptosis and may be simply a protein–protein interaction domain. Deletions between Bak and Bax show that the BH3 domain serves as a minimal ‘death domain’ critical for both dimerization and killing. The BH3 domain includes the BID, BAD, BIK, BIM, BLK and HRK and EGL-1 of C. elegans.

_bak_ is an accelerator of apoptosis and shows a direct link between viral reactivation of apoptosis (E1B19K) and bcl-2. bad is another member of this family and its product possesses the BH1 and BH2 domains. It interacts strongly with Bcl-xL but not with Bax. The first and only crystal and solution structure of Bcl-2 family members known is of Bcl-xL (ref. 88). It contains two hydrophobic central helices arranged in antiparallel fashions surrounded by five amphipathic helices. The three Bcl-2 homology region (i.e. BH1-3) is in close proximity forming a hydrophobic cleft with highly conserved Gly138 in BH1 domain. Bcl-xL is analogous to bacterial toxins in structure and has similarity in membrane insertion domains. By functional analogy they may form pores in the cytoplasmic and nuclear membranes and function by regulating signals dependent on pH voltage or ionic strength. They could form ion channels but this concept is still unclear.

**ICE family of cysteine proteases**

Cysteine proteases or caspases are the core of a conserved cell death pathway. In the nomenclature, caspase ‘c’ denotes a cysteine protease and ‘asparase’ refers to its ability to cleave an aspartic acid residue. Phylogenetic analysis of caspases reveals that there are three subfamilies:

(a) An ICE subfamily comprising caspase-1, -4 and -5.
(b) CED/CPP32 (32 kDa cysteine protease) subfamily comprising caspase-3, -6 to -10.
(c) ICH-1 comprising caspase-2.

Cloning of ICE cDNA revealed that the enzyme is synthesized as a 45 kDa proenzyme which is matured by proteolysis to yield a large (p20) and a small (p10) subunit. The crystal structure revealed that the active enzyme is a heterotetramer [[p20 p10]4]. The pairing face of the dimer is composed of p10 subunits while the catalytic site comprises residues from both the subunits. Cloning of the cDNA shows five different transcripts from the ICE gene, out of which two encode polypeptides which are catalytically inactive products. Mice lacking ICE develop normally but have defective IL-1β processing and Fas-mediated apoptosis.

PCR cloning strategies for the identification of related transcripts of ICE proved useful and now ten IRPs (ICE-related proteases, or caspases 1–10) have been identified. These include ICE, CED-3, Nedd2/Ich1, TX/ICErel1, Ich2, CPP32/YAMA, Mch-2 and ICErel III. This reveals an extended family of structurally related enzymes. The question which arises is why have so many IRPs? Different possibilities do exist but still clear proofs are needed; Some of them are:

(a) May be exhibiting expression in different tissues and cell types.
(b) May be the enzyme functions only in combinations.
(c) Probably enzymes work in parallel with one another or in a cascade.

A recent report on the hierarchical activation of the caspases has been shown by Slee et al. Exit of cyto-
chrome c from the mitochondria into the cytosol is an important step in apoptosis, where propagation of the death signal is by caspase-9 which triggers the other caspase activation events. There is a hierarchical activation of caspase-2, -3, -6, -7, -8 and -10 in a caspase-9 dependent manner\textsuperscript{91}.

The DNA repair enzyme poly (ADP-ribose) polymerase (PARP) was the first identified substrate for IRP during apoptosis which was identified using a cell-free system\textsuperscript{92}. PARP is cleaved by a proteolytic activity termed as a protease resembling ICE (prICE). prICE does not cleave pro IL-1β and purified ICE does not cleave purified PARP. The only other proven substrate for IRPs during apoptosis are the nuclear lamins, a family of intermediate filament proteins which form a meshwork that provides structural support for the inner nuclear membrane\textsuperscript{93,94}. The cleavage of lamin is accompanied by the disassembly of nuclear lamina, though not the nuclear envelope.

Mitochondria and PCD

The mitochondria participates in the cytoplasmic control of PCD and probably intervenes in the effector phase. The essential role of mitochondria is oxidative phosphorylation, in cellular energy production and generation of reactive oxygen species (ROS), and the initiation of apoptosis has suggested a number of novel mechanisms for the mitochondrial pathology. The importance and interrelationships of these functions are now being studied in mouse\textsuperscript{95}. Detection of changes in mitochondrial functions during apoptosis by simultaneous staining with multiple fluorescent drugs and correlated multi-parameter flow cytometry has now been well established\textsuperscript{96}.

The mitochondria plays a central role in apoptosis by release of cytochrome c and activation of caspase. Pro-caspase 3 is localized in the mitochondria in Jurkat cells in a complex with chaperone proteins Hsp60 and Hsp10 (ref. 97). It is known that mitochondria are essential for pro-caspase 3-21 complex formation to resist Fas-mediated cell death\textsuperscript{98}. Linsinger et al.\textsuperscript{99} suggest that uncouplers of oxidative phosphorylation can presensitize some but not all cells for a Fas-death signal and provide information about the existence of a separate pathways in the induction of apoptosis\textsuperscript{100}.

Proapoptotic molecular Bax and the constitutive mitochondrial protein adenine nucleotide translocator (ANT) cooperate within the permeability transition pore complex (PTPC) to increase mitochondrial membrane permeability and to trigger cell death\textsuperscript{100-102}. BID, an intracellular cross-talk agent that can amplify FAS/TNF apoptotic signal through the mitochondrial death pathway after caspase 8 cleavage induces conformational change of Bax which is responsible for release of cyto c from mitochondria during apoptosis\textsuperscript{103}. The structure of BID has also been established\textsuperscript{104}.

The Bax-induced release of cyto c in yeast does not involve any permeability transition of the inner mitochondrial membrane but involves a general alteration of the permeability of the outer mitochondrial membrane to macromolecules. It suggests that permeability transition of the inner mitochondrial membrane is not an event required for relocation of cyto c in yeast. The outer membrane voltage-dependent anion channel (VDAC), a putative component of the permeability transition pore, is not involved in Bax-induced release of cyto c or in the prevention of this release by Bcl-XL. Bax devoid of its C-terminal putative hydrophobic α-helix is as efficient as the full length Bax and allows the relocation of cyto c, demonstrating that this segment of the protein is not required for membrane targeting. Action of Bax on the outer mitochondrial membrane requires the presence of ATP both in vitro and in vivo and it is shown that ATP directly increases the amount of Bax inserted into the mitochondria\textsuperscript{105}. A cytosolic factor is required for mitochondrial cyto c efflux during apoptosis\textsuperscript{106}.

It has been shown using inhibitors that mitochondrial permeability transition causes intracellular acidification.

Release of two mitochondrial proteins, cyto c and apoptosis-inducing factor (AIF) into soluble cytosol of cells undergoing apoptosis is well established. Only intermembrane proteins are released from the mitochondria during the early phase of the apoptotic process\textsuperscript{107}. The molecular characterization of AIF has already been done\textsuperscript{108}. AIF is a 57 kDa flavoprotein having homology with bacterial oxidoreductase and is sufficient to induce apoptosis in isolated nuclei. It is confined to the mitochondria but translocates to the nucleus when apoptosis is induced. Bax, a proapoptotic member of Bcl-2 family translocates from the cytosol to the mitochondria during PCD. Both gain of function and loss of function mutations can be achieved by altering a single amino acid in the Bax hydrophilic C-terminus. The properly mutated C-terminus of Bax can target a non-relevant protein to the mitochondria showing that a specific conformation of this domain alone allows mitochondrial docking. These data along with N-terminus epitope exposure experiments suggest that the C- and N-terminals interact and that upon triggering of apoptosis Bax changes conformation, exposing these two domains to insert into the mitochondria and regulate the cell death machinery\textsuperscript{109}. Another report\textsuperscript{110} shows that the antiapoptotic effort of Bcl-2 can be overcome by phosphorylation of Ser 70. Forms of Bcl-2 lacking the loop region are much more effective at preventing apoptosis than wild type Bcl-2 because they cannot be phosphorylated.

A reduction in mitochondrial transmembrane potential (ΔΨm) occurring before nuclear changes in PCD is a constant observation in rather different types of PCD. Reduction of ΔΨm correlates with a decrease in mitochondrial translation and a loss in mitochondrial gene transcription.

Findings suggest that DNA damage by duocarmycin A induces H2O2 generation which causes mitochondrial
membrane potential loss and subsequently caspase 3 activation resulting in apoptosis.\(^\text{111}\)

The evolution of a mechanism of cell suicide has been well illustrated by Blackstone and Green\(^\text{112}\). In vertebrates, PCD or apoptosis frequently involves a relocalization of mitochondrial cyto c to the cytoplasm. This role in regulation of apoptosis is in addition to the primary function of cyto c in mitochondrial electron transport chain. These divergent roles became plausible on considering the symbiotic origin of the mitochondria. Symbiosis involves conflicts between levels of selection, in this case between primitive host cell and protomitochondria. In an aerobic environment selection on the protomitochondria may have favored routine manipulations of the host cell’s phenotype using products and by products of oxidative phosphorylation in particular ROS. Blocking the mitochondrial electron transport chain by removing cyto c enhances the production of ROS, thus cyto c release by protomitochondria may have altered the host cell’s phenotype via enhanced ROS production. Subsequently, the signalling pathway may have been refined by relation so that cyto c itself became the trigger for changes in the host’s phenotypes. A mechanism of apoptosis in metazoans may thus be a vestige of evolutionary conflicts within the eukaryotic cell.

**Death receptors**

So far, three factors tumour necrosis factor (TNF), Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) as well as four death factor receptors (Fas, TNFRI, DR3/Ws1-1 and CAR1) have been identified. It is speculated that many more death factor and receptor systems that regulate apoptosis in a tissue-specific manner would be found. The apoptotic signals triggered by death factors are irreversible, that is a protease cascade is activated by the death signal, and the proteases cleave various cellular components leading to morphological changes of the cell and nuclei that are typical for apoptosis.

Cytokines are a family of proteins that regulate cellular proliferation and differentiation by binding to their specific receptors on target cells. FasL or TNF bind to their receptors and induce apoptosis killing the cells within a few hours. FasL belongs to the TNF family\(^\text{113}\) which includes TRAIL. FasL is synthesized as a type II membrane protein, that is its N-terminal is in the cytoplasm and its C-terminal is in the extracellular space. The extracellular region of about 150 amino acids is well conserved (20-25%) among members of the TNF family while the length and the sequence of the cytoplasmic segments differ significantly. Proteolysis of membrane-associated TNF produces soluble TNF and the functional form of it exists as trimers.\(^\text{114}\)

Fas, (also known as APO-1 or CD 95) the receptor for FasL, is a type I membrane protein\(^\text{115}\) and a member of the TNF receptor (TNFR) family. This is a fast-growing family and many new members are being identified. The extracellular region of the TNF receptor family members carries 2-6 repeats of a cysteine-rich subdomain that has approximately 25% similarity among various members\(^\text{116}\). TNF induces apoptosis and activates the transcription factor NF-kB. It can also stimulate the proliferation of thymocytes. The presence of a homologous domain of about 80 amino acids in the cytoplasmic regions of Fas and TNFR1, suggests that possibly this could be responsible for transducing the death signal. This region has been designated as the ‘death domain’\(^\text{117}\). NMR spectroscopy suggests that the death domain is a novel protein fold consisting of six anti-parallel, amphipathic α-helices\(^\text{118}\). The charged amino acids present on its surface are probably responsible for mediating the interactions between the death domains. The binding of the ligands to the above said receptors induces trimerization of the receptors. This trimerized cytoplasmic region then transduces the signal. Fas and TNFRI mediated apoptosis occurs in the presence of inhibitors of protein and RNA synthesis\(^\text{119}\) suggesting that all the necessary components for apoptotic signal transduction are present and that the activation simply triggers the machinery.

Fas-associated protein with death domain (FADD) or MORT 1 which contains a death domain, and its C-terminus\(^\text{119}\) is recruited to Fas upon its activation and binds to Fas via interactions between death domains. The N-terminal region, called the death effector domain (DED) or MORT 1 domain, is responsible for the downstream signal transduction. A similar death domain-containing protein TNFRI associated death domain protein (TRADD), binds to TNFRI\(^\text{121}\) but does not carry a DED and its death domain is responsible for mediating apoptosis. This discrepancy was resolved by finding that TRADD binds to FADD/MORT 1 via interactions between their death domains.\(^\text{122}\) Results suggest that Fas and TNFRI use FADD as a common signal transducer and share the signalling machinery downstream of FADD/MORT 1. In addition to this, TNFRI has another pathway leading to apoptosis. Receptor interacting protein (RIP) is a serine/threonine kinase containing a death domain and binds to TRADD via interactions between death domains and induces apoptosis when overexpressed. RIP does not have DED, therefore, another downstream effector molecule may be recruited through its death domain for the transduction of the death signal. The signalling molecule downstream of FADD/MORT 1 was identified as FLICE (FADD like ICl) or MACH (MORT 1-associated CED-3 homologue) as is now called as caspase-8 (ref. 123). Caspase-8 carries two DED/MORT 1 domains at the N-terminus through which it binds to FADD/MORT 1. The C-terminal region is related to the ICE family members. It is likely that other members of the ICE family are also activated in the cascade, cleaving their ‘death substrates’, e.g. lamin, actin, poly (ADP) ribose polymerase, etc. to cause the apoptotic morphological changes observed in
cells and nuclei and chromosomal DNA degradation. Recently, it has been found that Fas-induced B cell apoptosis requires an increase in free-intracellular Mg²⁺ concentration as an early event. Nematode CED-4 protein and its human homologue Apaf-1 play a central role in apoptosis by functioning as direct activators of death-inducing caspases. CARD-4 (caspase recruitment protein) was identified to have a domain structure strikingly similar to cytoplasmic receptor-like proteins that mediate disease resistance in plants. CARD-4 coordinates downstream NF-kB and apoptotic signalling pathways and may be a component of the host innate immune response.

Cross-talk

The entrance to the core death pathway is tightly regulated. The regulation of cell death is possibly as complicated as the regulation of cell survival and proliferation. Similar to the mitotic pathway the death pathway is also influenced by multiple kinase cascades which may not be directly involved in the core death programme. Therefore, it is very important to categorize the kinases as those involved in signalling pathway, execution pathway or those which influence the death pathway.

No known kinase has been shown to be involved in bringing about the execution of the death pathway. It is the ICE family proteases which are known to be executioners of the death programme and none of them has been shown to be regulated by phosphorylation. Most of the known kinase cascades, only amplify the exogenous stimuli and integrate conflicting signals prior to entry to apoptosis.

MAP/ERK family kinases serve to regulate both cell proliferation and cell death. The RAS/RAF/MKK/ERK signalling pathways are engaged by a variety of mitogenic receptors mainly those whose cytoplasmic domains are tyrosine kinases. Upon ligand binding, the tyrosine-kinase is activated and results in the recruitment of guanine nucleotide exchange factors required for activation of RAS and liberation of lipid second messenger, i.e. diacylglycerol which again triggers the lipid kinase cascade.

The requirement for distinct lipid second messenger for the activation of growth-promoting and growth-inhibitory kinase cascades may provide the cell an opportunity to integrate the response to conflicting stimuli. It has been shown that the presence of diacylglycerol can profoundly influence the cellular response to ceramide and elevation of ceramide concentration is seen both in cells undergoing stress-induced apoptosis and those which are triggered for growth and differentiation. Diacylglycerol inhibits TNF-α and ceramide-induced apoptosis suggesting a role for cross-talk between these antagonistic lipid mediators. The precise role of lipid-activated kinases engaged in ceramide-induced death pathway is still not known but it definitely suggests an important role for lipid second messengers in integrating signals leading to cell growth or cell death. BCL-2, a potent inhibitor of apoptosis, interacts with both RAS and RAF suggesting that interactions between CED-9/BCL-2 family members and RAS/RAF/MKK/ERK signalling pathway are also activated during FAS-mediated apoptosis.

Another nucleic acid-dependent kinase that has been proposed to regulate apoptosis is DNA-PK, a nuclear serine/threonine kinase that is a substrate for ICE-like proteases activated during apoptosis. It is still unclear whether proteolysis of DNA-PK is required for apoptosis, the observation that proteolytic fragments retain kinase activity suggests the possibility that the substrate specificity is altered following cleavage. Since several DNA repair enzymes are cleaved by ICE family proteases during apoptosis, it has been proposed that cleavage of DNA-PK ensures the irreversibility of apoptotic cell death by preventing DNA repair. Thus it is clear that multiple serine/threonine kinases function as molecular gatekeepers guarding the entry into the core death pathway. Depending on the cell type and experimental systems it is possible that kinases can either enhance or inhibit susceptibility to apoptosis. The ability of parallel cascades to integrate proliferation and anti-proliferative signals requires that some kinases will affect both cell cycle progression and apoptosis.

PCD and oncogenesis

Prevention of PCD contributes to tumour formation in two ways: (a) Accumulation of unwanted cells can become tumourigenic by itself; (b) In the absence of cell death, cells live longer and become likely candidates to harbour oncogenic mutations resulting in tumour formation. Many oncogenes have the ability to affect cell death.

p53

The dominant as well as the recessive mutations in the tumour suppressor gene encoding p53 can be tumourigenic. p53 activates the transcription of Cip1/WAF1 protein, which blocks the activation of cyclin-dependent kinases. Association of cyclin and cyclin-dependent kinases is a must for the cells to enter the S phase. Apoptosis under certain conditions has been thought to be p53 dependent, for example, radiation-induced death. Experiments by Lowe et al. suggest that the cytotoxic effects of radiation and anticancer drugs are mediated through a p53-dependent pathway. The exact mechanism of activation of p53 in a cell and how it induces cells to die is not yet known. p53-dependent apoptosis is independent of new RNA or protein synthesis, indicating that p53 induces apoptosis without specific gene activation. It may repress genes necessary for cell survival or be a
component of the molecular machinery for execution of apoptosis.

p21

p21 is another gene associated with the p53 gene. It was discovered as a senescent cell-derived inhibitor of DNA replication in human diploid fibroblasts\textsuperscript{139}. It is induced in a p53-dependent G1 arrest in normal human diploid fibroblasts resulting in the inhibition of cdk2-cyclin E kinase activity, thereby blocking its entry into the S phase\textsuperscript{140}.

ras

Three variations of p21ras proteins (Ha-ras, ki-ras and N-ras) identified are key signal transducers\textsuperscript{141}. They promote cell proliferation and oncogenesis of proliferating cells and can also mimic the functions of NGF. Survival of neurons is NGF-dependent. It is believed that ras may be involved in the intracellular signalling pathway for survival effects of neurotrophic factors. A part of the amino acid of p21ras has a homology with cystatins, an endogenous cysteine protease inhibitor\textsuperscript{142}. Probably ras-related proteins regulate Bel-2 proteins and prevent cell death because of being cysteine protease inhibitors\textsuperscript{143,144}.

c-myc

c-myc expression is induced by mitogenic stimulation. c-myc is down-regulated and cells get arrested in G1 phase following removal of serum from culture media\textsuperscript{145}. Expression of endogenous c-myc is required for apoptosis induced by CD3/TCR stimulation. The same region of c-myc is required for transformation, transcription activation and induction of apoptosis which implies a close relationship between cell proliferation, transformation and apoptosis as a function of c-myc\textsuperscript{146,147}.

c-fos

Expression of the c-fos proto-oncogene has been associated with a variety of biochemical responses, including proliferation, differentiation and neural excitation\textsuperscript{148}. c-fos is very similar to c-myc where its continuous expression renders cells more sensitive to apoptotic signals and the signal transduction machinery of cell death may be shared with cell proliferation and differentiation. Both c-fos and c-myc can be induced by oxidative stress\textsuperscript{149}.

c-rel

c-rel is a transcription factor and is a member of NF-kB family. The highest level of expression is detected in cells undergoing PCD\textsuperscript{150}. Overexpression of c-rel in primary avian fibroblast cells results in extended life spans. Thus, it is possible that like any other cellular oncogenes c-rel can also regulate both cell proliferation and cell death.

Evolutionary origin of PCD

The very first question which comes to our minds is where exactly did this death programme originate – in plants or in the animals? From the data available it is difficult to correlate the death programmes in plants as the molecular mechanism(s) of plant cell death is still not understood. In animals, is it the unicellular or the multicellular organisms which showed this phenomenon?

The most convincing cell death programmes in unicellular organisms are seen in prokaryotes where they arose as a result of competition between bacteria themselves, between viruses (one strain vs another strain) or among bacteria and viruses. The molecular mechanisms involved are varied\textsuperscript{151}. Some of them work by cleaving the RNA, DNA or some specific proteins. There are some similarities between one of the suicide programmes of E. coli with the PCDs of animal cells. It is based on a constitutively expressed proteolytic enzyme, which gets activated in response to T4 phage infection. After binding, the enzyme cleaves the translational elongation factor, arresting translation and thereby killing the cell\textsuperscript{152}. In other words, it commits suicide in response to infection, thereby protecting its nearest neighbouring cell. It could be possible that mechanisms like this in the ancestral prokaryote may have provided the starting point for the initiation of death programmes in animal cells.

Eukaryotic organisms, which remain unicellular throughout their life, for example, Trypanosomes, elicit death programmes with characteristics of apoptosis\textsuperscript{153}. The picture seems to be different with yeast, which under normal circumstances does not display any death programme. But the expression of death genes kills the cells. This can be blocked by coexpressing a death-suppressor gene\textsuperscript{154}. Cellular slime molds which are thought to have descended from an ancestral eukaryote, gave rise to plants, fungi and animals\textsuperscript{155}. They show both unicellular and multicellular forms in their life cycle, a typical example is Dictostelium discoideum. They display two terminally differentiated cell types, the viable spore cells and the dead vacuolated stalk cells. Stalk cells die as a normal part of their differentiation programme and display features quite similar to apoptotic animal cells\textsuperscript{5}. The molecular mechanism of its cell death programme is still not known\textsuperscript{156}.

Cell death plays a crucial role in plant development\textsuperscript{157} and shares similarities in morphological features with PCDs in animals\textsuperscript{158}. The dead cells cannot be phagocytosed in plants because of the presence of a thick cell wall. This mystery still remains unsolved.
Applications

The apoptosis technology has great commercial asset as its implications for diseases where it is the cause for pathology (example Alzheimer's and Parkinson's diseases), immunosuppression or an effect of trauma (example stroke and heart attack). Pathological suppression is also important for cancer and viral infections. Another important aspect is not in the disease and its treatment but the maintenance of several industrially useful cell lines (e.g., for production of antibodies and vaccines) which require costly growth factors. They could be maintained for longer times with less effective cell deaths by altering the genetic make-up of these cells. In other words, manipulation of such genes could be used to modify permanently the apoptotic tendency of these cell lines.

Cancer is a disease with excessive cell proliferation and insufficient cell death. The first thing would be to kill the tumour since some cells could be cancerous, particularly some lymphoid tumours express functional Fas. In order to avoid side-effects, methods need to be employed with local administration or proper targeting of Fas-L to the target regions. Fas have been shown to play an important role in human diseases like AIDS, hepatitis, etc. Neutralizing antibodies against them (may be by an inhibitor) would have potential as therapeutic agents. Researches on this approach are being carried out extensively. What is needed is the proper targeting to the area specified or the use of inhibitors in the particular cascade of apoptosis.

Conclusions

Many growth and differentiation factors regulate proliferation and differentiation during development of an organism. One would expect to find many more death factors and receptor systems which could regulate PCD at the organissimal level as well as in a tissue-specific manner. Signals for growth and differentiation mediated by phosphorylation and diphosphorylation of proteins or by small second messengers are generally found to be reversible while the signals for PCD are irreversible.

The structural and functional similarities of Ced-9 and Bcl-2 and Ced-3 and ICE suggest that there may be common molecular pathways of PCD in all metazoans. Though now we find that as we go up the evolutionary tree the system gets complicated as at least ten members of the caspase family and nine members of the Bcl-2 family have been identified. Further evidences are needed to determine the precise role of these genes in the control of cell death and in other pathways also.

The molecular biology of apoptosis is considerably more advanced than the cell biology of apoptosis. It is important to know their subcellular localizations and their interactions with one another in the cell and also with signals from other cells.


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