Hypoxia, ischaemia, and apoptosis

Apoptosis, meaning "the falling of leaves," was coined in 1976 by Kerr, Wyllie, and Currie to describe tumour cells undergoing a different and unusual mode of death, with the implication that these cells were dying quietly, like autumn leaves. It is now understood that these apoptotic cells were demonstrating a physiological cell death that is ubiquitous and essential to the development and survival of multicellular organisms. This mode of death involves the activation of an intrinsic, constitutive "death programme" that can be triggered by multiple stimuli, including loss of trophic factors, activation of specific membrane receptors, viral infection and cellular injury resulting from insults such as hypoxia-ischaemia or ionising radiation. Apoptotic death is distinct from necrosis, requiring time, energy, and in some cases, gene transcription and translation.

Apoptosis and necrosis
Apoptosis was originally distinguished from necrosis on morphological grounds. Cells undergoing necrosis due to an overwhelming insult (such as complete mitochondrial inhibition), swell as their membrane pumps fail to maintain ionic homeostasis in a process that does not require energy or gene transcription. Organelles become disrupted and finally the cell bursts, spilling the cytoplasmic contents into the extracellular space. Phagocytes migrate to the site of injury and remove the debris with all the hallmarks of a classic inflammatory response.

Apoptotic cells undergo a very different process. They shrink and the nucleus becomes small and dense. The DNA breaks into discrete masses and the nuclear membrane folds around the pieces like shrink wrap. At the same time, the plasma membrane invaginates, resulting in cytoplasmic vacuolation. Eventually the cell separates into multiple discrete apoptotic bodies which are phagocytosed primarily by healthy neighbouring cells. The lack of an inflammatory response and the fact that apoptosis occurs physiologically during normal development gives an impression of individual cells which die altruistically for the good of the whole organism. Apoptosis is an active cellular process that requires specific biochemical pathways, energy consumption, and sometimes gene transcription.

Worms and death
Fittingly perhaps, the cellular machinery of the apoptotic death programme began to be understood largely through studies of a worm—the nematode Caenorhabditis elegans. This convenient organism has only 1090 cells of which 131 undergo apoptosis during normal development. As the worms are transparent, the cells can be viewed as they divide, differentiate, and die. In elegant studies of genetic mutants Horvitz and colleagues were able to define three genes (the C elegans death, or ced family), involved in this developmentally programmed cell death. Disruption of any of these genes produced either lethal or highly abnormal phenotypes.

Two genes, ced-3 and ced-4, are essential for the execution of the apoptotic programme. Ced-3 encodes a cysteine protease that shares a significant degree of homology with interleukin-1β converting enzyme (ICE), a protease that cleaves pro-interleukin-1β into active interleukin-1β. ICE can induce apoptosis when overexpressed in neuronal cells. The function of ced-4 is not yet clearly defined. The third gene, ced-9, prevents apoptosis, and is homologous to the mammalian gene bcl-2, which is implicated in the pathogenesis of B cell cancers. Indeed, bcl-2 can substitute ced-9 in mutant worms and rescue the phenotype from excess apoptosis.

These discoveries demonstrated that apoptosis is a highly conserved, genetically regulated process that is fundamental to normal development in organisms as diverse as worms and humans.

Death by default: apoptosis in the multicellular organism
Insight into the widespread importance of programmed cell death came from the demonstration by Raff that cells will always undergo apoptosis unless they receive "survival signals" from other cells. They showed that cells in culture would universally undergo apoptosis unless either they were maintained at high density or the culture medium was artificially supplemented with appropriate trophic factors. This suggested that proteins, normally growth factors such as insulin-like growth factor-1 (IGF-1) or members of the neurotrophin family, are secreted in an autocrine or paracrine manner, and are essential to prevent execution of the apoptotic death programme. If cells are programmed to die as soon as they lose these survival signals, the surprising conclusion must be that death, not survival, is the default pathway for cells in a multicellular organism.

The death of cells deprived of signals from their neighbours provides an elegant mechanism for both the control of normal morphogenesis and the prevention of cancers: a cell population which grows beyond its "correct" size or location will run out of trophic factors and die. A striking example is the formation of digits from the primitive limb bud. Interdigital cells in the chick limb bud die by apoptosis at a precisely controlled stage of development under the control of growth factors of the transforming growth factor β family. Chickens thus develop feet with digits; ducks, which retain webbed feet, do not express these growth factors and do not develop interdigital apoptosis.

Brain cells which fail to make proper synaptic connections die by apoptosis and as many as 50% of cells in some parts of the brain may die in this way during normal cerebral development. Apoptosis is thus the chisel that sculpts the complex organism. In newborn infants developmental processes that involve the apoptotic death of unwanted cells are at their height, and cells may be particularly susceptible to apoptosis at this age.

Apoptosis in disease
In addition to being a primary mechanism for regulating normal development, apoptosis is involved in the patho-
genesis of many diseases. An increasing number of disorders have been identified that involve excess apoptotic cell death. These include AIDS, where lymphocyte death is triggered by HIV; neurodegenerative disorders, such as spinal muscular atrophy and Parkinson's disease; and hypoxic-ischaemic cerebral damage.10

Other disorders are associated with increased cell survival, including certain autoimmune diseases, viral infections, and particularly cancer. The multicellular organism requires stringent protection against the development of tumours, and apoptosis forms a front line defence. Cells which grow abnormally rapidly are likely to run out of growth factors and be eliminated by apoptosis. Cellular injury, including tumorigenic DNA damage, can trigger the innate immune death programme. However, if an insult creates a mutation that results in the activation of anti-apoptotic genes such as bcl-2, these cells will acquire a survival advantage that could promote carcinogenesis. Cancers can no longer be regarded solely as diseases of deregulated cell division, but also as the result of a failure in apoptotic cell removal.11

**Triggering apoptosis**

Apoptosis can be activated by many physiological and pathological stimuli: trophic factors such as IGF-1 bind to tyrosine kinase receptors, and their withdrawal probably kills by removing essential phosphorylation signals; DNA damage can lead to the activation of the p53 tumour suppressor gene which can drive the cell to apoptosis;2 cells in the immune system undergo apoptosis when activated by a cell surface receptor, Fas/Apo-1;13 tumour necrosis factor-α (TNF-α) activates sphingomyelinase, leading to an increase in cytosolic concentrations of ceramide, a potent inducer of apoptosis.14 Some agents kill by triggering unknown mechanisms: for example, different rebox forms of nitric oxide induce apoptosis by different mechanisms, only one of which involves free radical activity.15

**Mechanisms of apoptosis**

The intracellular mechanisms of apoptosis have not been fully elucidated, although an intimate connection with control of the cell cycle is evident in carcinogenesis: oncogenes such as c-fos, c-jun, and c-myc can trigger apoptosis and promote cell division while inactivation of the pro-apoptotic p53 tumour suppressor gene is a frequent marker of human neoplasia.16 If death is the default pathway of all cells, then the apoptotic machinery must be permanently in place and poised to act; indeed, in many cases apoptosis can proceed in the absence of de novo messenger RNA or protein synthesis. Execution of this pathway differs between cell types and involves poorly understood second messengers such as accumulation of calcium or cyclic AMP, or activation of protein kinase C.17 One of the earliest signals in the apoptotic cascade is the activation of specific cysteine proteases, such as ICE and casp-3. These enzymes are activated very soon after the death trigger and cleave specific cellular targets, such as poly-ADP-ribose polymerase.18 A common feature of final apoptotic execution is the degradation of genomic DNA by an endonuclease. This leads to internucleosomal cleavage of DNA into fragments differing by 200 base pairs, producing characteristic “ladders” on gel electrophoresis.19

**Apoptosis in hypoxic-ischaemic cerebral injury**

It has been known for some years that newborn infants subjected to transient hypoxia-ischaemia during an episode of birth asphyxia are apparently relatively normal soon after resuscitation, but show evidence of delayed cerebral injury some hours later,20 the magnitude of which predicts the severity of later neurodevelopmental impairment.21 This biphasic process of cerebral injury has been reproduced in several animal models.22,23

The mechanism of delayed injury is unclear, but in the brains of infants dying after birth asphyxia cells can be detected which show the hallmarks of apoptotic death: nuclear condensation and fragmentation (pyknosis and karyorrhexis) with preservation of the plasma and nuclear membranes.24 This has led to the hypothesis that inappropriate activation of the apoptotic programme accounts for at least part of the delayed cell death seen after transient hypoxia-ischaemia.

Evidence is now accumulating to support this contention. The features of apoptosis can be observed following hypoxic-ischaemic injury in newborn piglets,24,25 and in both immature26,27 and adult rats,28,29 as well as in studies of in vitro models of neuronal hypoxia.30 In newborn piglets which develop delayed cerebral injury after hypoxia-ischaemia a large number of cells are apoptotic, and there is a linear relation between the severity of the hypoxic-ischaemic injury (measured by the decline in cerebral ATP concentrations during hypoxia-ischaemia) and the proportion of apoptotic cells seen 48 hours later.31

The mechanism by which transient hypoxia-ischaemia induces apoptosis is unknown. It could be several different or overlapping pathways, including DNA damage, increases in intracellular calcium concentrations or other forms of intracellular disorganisation. Survival signals might be reduced by impaired secretion from injured cells, damage to membrane receptors, or interruption of intracellular signalling pathways. Known agents of hypoxic-ischaemic damage such as glutamate or nitric oxide can under some conditions induce apoptosis,32 suggesting that cells which avoid rapid necrosis during the initial insult may instead be triggered to undergo apoptosis some hours later.

Significant numbers of necrotic cells are also present after hypoxia-ischaemia, but the distinction between these two modes of death is demonstrated in newborn piglets by differences in the response to therapeutic manipulations. A mild (3°C) reduction in brain temperature in the 12 hours following resuscitation significantly reduced the observed proportion of apoptotic cells but made no difference to the amount of necrosis.33

**Therapeutic inhibition of apoptosis**

Anti-apoptotic agents reduce neuronal loss following experimental cerebral hypoxia-ischaemia, suggesting that (for a time at least) the apoptotic process is reversible: both IGF-1 and the protein synthesis inhibitor cycloheximide, reduce cerebral injury if administered soon after the insult.34 Hypothermia reduces apoptosis by an unknown action, presumably related to the reduction of some relevant aspect of cellular metabolism which prevents the cascade from reaching completion.35 The evidence that apoptosis triggered by hypoxia-ischaemia may be interrupted suggests that anti-apoptotic agents or strategies might be useful neural rescue therapies when administered soon after a hypoxic-ischaemic insult.

**Conclusion**

Rapid progress has been made in understanding the mechanisms of cell death, and an accumulating body of evidence indicates a major role for apoptosis in disease. Apoptosis is a highly regulated physiological process, which suggests new avenues for research into therapies for hypoxic-ischaemic cerebral injury and other pathological processes. As the molecular mechanisms of apoptosis were
unravelled, it should become possible to design precise experiments to implement these findings in clinically applicable ways.

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