Annotation

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.¹ 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.² 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.³

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.³ 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.⁴

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-1 β converting enzyme (ICE), a protease that cleaves pro-interleukin-1 β into active interleukin-1 β . ICE can induce apoptosis when overexpressed in neuronal cells.⁵ 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.⁶ Indeed, *bcl-2* can substitute *ced-9* in mutant worms and rescue the phenotype from excess apoptosis.⁷

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.² 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.⁸ 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.⁹ 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 intrinsic 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 signals²; DNA damage can lead to the activation of the p53 tumour suppressor gene which can drive the cell to apoptosis¹²; cells in the immune system undergo apoptosis when activated by a cell surface receptor, Fas/Apo-1¹³; 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 other, unknown mechanisms: for example, different redox 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 *ced-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,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.²⁵

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,³¹ 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.³²

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.³³⁻³⁴ 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.³² 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 are

unravelling, it should become possible to design precise experiments to implement these findings in clinically applicable ways.

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- 1 Kerr JF, Wyllie AH, Currie AR. Apoptosis, a basic biological phenomenon with wide-ranging implications in human tissue kinetics. *Br J Cancer* 1972; 26:239-57.
- 2 Raff MC. Social controls on cell survival and cell death. *Nature* 1992;356:397-400.
- 3 Wyllie AH, Duvall E. Cell injury and death. In: McGee JO'D, Isacsson PG, Wright NA, eds *Oxford textbook of pathology*. Oxford: Oxford University Press, 1992:141-93.
- 4 Osborne BA, Schwartz LM. Essential genes that regulate apoptosis. *Trends Cell Biol* 1994;4:394-8.
- 5 Miura M, Zhu H, Rotello R, Hartweg EA, Yuan J. Induction of apoptosis in fibroblasts by IL-1 beta converting enzyme, a mammalian homologue of the c-elegans cell death gene ced-9. *Cell* 1993;75:653-60.
- 6 Hengartner MO, Horvitz HR. C.elegans cell survival gene ced-9 encodes a functional homologue of the mammalian proto-oncogene bcl-2. *Cell* 1994; 76:665-76.
- 7 Vaux DL, Weissman IL, Kim SK. Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2. *Science* 1992;258:1955-7.
- 8 Zou H, Niswander L. Requirement for BMP signalling in interdigital apoptosis and scale formation. *Science* 1996;272:738-41.
- 9 Oppenheim RW. Cell death during development of the nervous system. *Ann Rev Neurosci* 1991;14:453-501.
- 10 Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995;267:1456-62.
- 11 Thomas NSB. *Apoptosis and cell cycle control in cancer*. Oxford: BIOS, 1996.
- 12 Yonish-Rouach E, Resnitsky D, Lotem J, Sachs L, Kimchi A, Oren M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* 1991;352:345-7.
- 13 Schulze-Osthoff K. The Fas/APO1 receptor and its deadly ligand. *Trends Cell Biol* 1994;4:421-7.
- 14 Hannun YA, Obleid LM. Ceramide: an intracellular signal for apoptosis. *Trends Biochem Sci* 1995;20:73-7.
- 15 Khan S, Hughes MN, Edwards AD, Mehmet H. The effect of nitric oxide and the nitrosonium cation (donated by sodium nitroprusside) on apoptosis in Swiss 3T3 fibroblasts. In: Moncada S, Stamler JS, Gross S, Higgs EA, eds. *The biology of nitric oxide (5)*. Colchester:Portland Press, 1996:133-6.
- 16 Evan GI, Harrington E, McCarthy N, Gilbert C, Benedict MA, Nunez G. Integrated control of cell proliferation and apoptosis by oncogenes. In: Thomas NSB, ed. *Apoptosis and cell cycle control in cancer*. Oxford: BIOS, 1996:109-29.
- 17 McConkey DI, Orrenius S. Signal transduction pathways to apoptosis. *Trends Cell Biol* 1994;4:370-5.
- 18 Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 1994;371:346-7.
- 19 Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980;284:555-6.
- 20 Azzopardi D, Wyatt JS, Cady EB, et al. Prognosis of newborn infants with hypoxic-ischemic brain injury assessed by phosphorus magnetic resonance spectroscopy. *Pediatr Res* 1989;25:445-51.
- 21 Roth SC, Edwards AD, Cady EB, et al. Relation between cerebral oxidative metabolism following birth asphyxia and neurodevelopmental outcome and brain growth at one year. *Dev Med Child Neurol* 1992;34:285-95.
- 22 Lorek A, Takei Y, Cady EB, et al. Delayed ('secondary') cerebral energy failure following acute hypoxia-ischaemia in the newborn piglet: continuous 48-hour studies by 31P magnetic resonance spectroscopy. *Pediatr Res* 1994;36:699-706.
- 23 Blumberg RM, Cady E, Wigglesworth JS, Edwards AD. Delayed impairment of cerebral energy metabolism following transient focal hypoxia-ischaemia in the 14 day old rat. *Exp Brain Res* (in press).
- 24 Yue X, Mehmet H, Squier MV, Hope PL, Azzopardi D, Edwards AD. Apoptosis and necrosis in the brains of infants dying after birth asphyxia. *Pediatr Res* 1995;37:387A.
- 25 Mehmet H, Yue X, Squier MV, et al. Increased apoptosis in the cingulate sulcus of newborn piglets following transient hypoxia-ischaemia is related to the degree of high energy phosphate depletion during the insult. *Neurosci Lett* 1994;181:121-5.
- 26 Ferrer I, Tortosa A, Macaya A, et al. Evidence of nuclear DNA fragmentation following hypoxia-ischaemia in the infant rat brain, and transient fore-brain ischaemia in the adult gerbil. *Brain Pathol* 1994;4:115-22.
- 27 Beilharz E, Williams CE, Dragunow M, Sirimanne E, Gluckman PD. Mechanisms of cell death following hypoxic-ischaemic injury in the immature rat: evidence of apoptosis during selective neuronal loss. *Mol Brain Res* 1995;29:1-14.
- 28 Linnik MD, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischaemia in rats. *Stroke* 1993;24:2002-8.
- 29 MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston E. Global ischaemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci Lett* 1993;164:89-92.
- 30 Rosenbaum DM, Michaelson M, Batter DK, Doshi P, Kessler JA. Evidence for hypoxia-induced, programmed cell death in cultured neurons. *Ann Neurol* 1996;36:864-70.
- 31 Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A* 1995;92:7162-6.
- 32 Edwards AD, Yue X, Squier MV, et al. Specific inhibition of apoptosis after cerebral hypoxia-ischaemia by moderate post-insult hypothermia. *Biochem Biophys Res Commun* 1995;217:1193-9.
- 33 Gluckman PD, Klempt N, Guan J, et al. A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochem Biophys Res Commun* 1992;182:593-9.
- 34 Goto K, Ishige A, Sekiguchi K, et al. Effects of cycloheximide on delayed neuronal death in rat hippocampus. *Brain Res* 1990;534:299-302.