Apoptosis in neural development and disease

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Introduction

Neural cell death has a pivotal role in both the development and pathophysiology of the nervous system. Two distinct modes of cell death—necrosis and apoptosis—are involved in pathological neuronal loss, but apoptosis alone is the mechanism of programmed cell death during development. All cells will undergo apoptosis in the absence of survival signals, usually peptide growth factors secreted by other cells. This provides an elegant mechanism for the control of neuronal development: a surplus of neurons is produced, and only those that form the correct connections with the target tissue receive adequate survival factors. The remainder undergo apoptotic death and removal. Apoptosis continues throughout life and is the central mechanism for the removal of surplus, unwanted, damaged or aged cells. Dysregulation of apoptosis is seen after cellular insults or in neurodegeneration as well as in tumourigenesis. Strategies which influence the apoptotic pathway offer valuable therapeutic approaches in a variety of pathological states.

Apoptosis

The term programmed cell death was first used by Lockshin and Williams in 1964, to describe the pre-determined loss of specific cells during insect metamorphosis by an intrinsic cellular suicide programme.¹ In a seminal paper Kerr, Wyllie, and Currie² later coined the word apoptosis to describe this form of death and went on to show that it was a widespread process in nature, occurring both during normal physiological development and in many pathological conditions.

Apoptosis is a well conserved and highly regulated mechanism of cell death for the removal of unnecessary, surplus, aged or damaged cells. Dysregulation of apoptosis can result in the persistence of mutated cells, leading to malformations, autoimmune disease, and cancer. On the other hand, inappropriate apoptosis resulting in the removal of healthy cells can occur in diseases such as infection, hypoxic-ischaemic injury, neurodegenerative or neuromuscular diseases, and AIDS.

Apoptosis can be distinguished from necrotic cell death³: in necrosis, a cell's demise is precipitated by an external insult and involves the early loss of membrane integrity, with damage to organelles and the leakage of cytoplasmic contents, leading to the recruitment of phagocytes with an acute inflammatory reaction. In contrast, apoptosis is cellular suicide. Cells dying by apoptosis retain membrane and organelle function until late in the process, while developing plasma membrane blebbing, reduced cytoplasmic volume, chro-

matin condensation and nuclear fragmentation. In the final stages cellular fragments wrapped in plasma membrane bud off as apoptotic bodies which are subsequently phagocytosed by healthy neighbouring cells.⁴ The elimination of cell debris occurs in the absence of an inflammatory response, and this quiet, rapid, and efficient removal of apoptotic cells means that apoptosis can be difficult to detect in tissue: although as many as 50% of the cells in a developing organ may undergo apoptosis, less than 1% of cells are apoptotic at any one time.

Apoptosis in the developing nervous system

Programmed cell death by apoptosis occurs in many developmental processes, including body sculpting (such as digit formation), elimination of self-reacting immune cells, sexual organ development and gamete formation.⁵ Indeed, a general principle of development in multicellular organisms is emerging: excess numbers of cells are made, and then surplus or unwanted cells are removed by apoptosis during the formation of functional organs.

In the developing nervous system apoptosis is observed as early as neural tube formation and persists throughout terminal differentiation of the neural network. The emergence of the neurotrophic hypothesis from the pioneering work of Levi-Montalcini, Hamburger, and Cohen⁶ established that more than 50% of neurons are lost during development as a result of limiting trophic support from the target tissue they are destined to innervate. This neuronal attrition, which ensures appropriate connections in the central and peripheral nervous systems, was subsequently shown to be effected by the induction of apoptosis in unwanted cells.

A growing number of neurotrophic factors, such as the nerve growth factor family, the neurokines, and growth factors such as insulin-like growth factors (IGF-I and IGF-II) support the survival of particular types of neurons.⁷⁻¹⁰ Targeted disruption of genes encoding these factors or their receptors indicates that particular neurotrophic factors are important for the development of specific neuronal populations.¹¹

Neurotrophic factors function by binding to specific receptors in the cell membrane, and the effects of NGF offer an example of the subtle control that the system allows. The nerve growth factor receptor has high and low affinity components. It will function as a survival factor if it binds to the high affinity trkA receptor, but will induce apoptosis of retinal neurons¹² or oligodendrocytes¹³ when it binds to the low affinity receptor p75 in the

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Figure 1 A model of apoptosis in neural cells. Neurotrophin withdrawal leads to death by a default mechanism through as yet uncharacterised pathways. Gene mutations, inappropriate production of neurotransmitters, such as glutamate, free radical generation, and mitochondrial damage also trigger apoptosis as do engagement of death receptors. In the latter case oligomerisation of Fas/Apo-1 or TNF receptors or the p75 NGF receptor results in the recruitment of death domain proteins, which in turn activate members of the caspase family of cysteine proteases in an organised proteolytic cascade. Bcl-2 family members and inhibitor of apoptosis proteins (IAPs) control the decision to commit cell suicide, depending on whether pro- or anti-apoptotic partners predominate. In the later stages, specific endonucleases cleave DNA as part of the packaging process. Finally, cells are phagocytosed by healthy neighbours (not shown). Arrows indicate the flow of events and do not necessarily denote direct interactions.

absence of trkA. Nerve growth factor in the extracellular environment is thus able to control neural development by both promoting growth of some cell types and the elimination of others.¹⁴

In some cases, however, targeted genetic disruption of neurotrophic factors or their receptors can leave the central nervous system apparently unaffected, suggesting that these factors are functionally redundant.¹⁵ It is now evident that the control of neuronal survival does not only depend on the provision of trophic molecules by the targets but also on activity, humoral factors, and trophic support from glia.¹⁶

It is not only neurons that undergo programmed cell death during differentiation. Apoptosis seems to regulate cell numbers in systems as diverse as the disappearance of the germinal layer during the third trimester of pregnancy¹⁷; the sexual differentiation of the medial preoptic nucleus, where apoptosis is controlled by testosterone¹⁸; lineages of the olfactory epithelium¹⁹; oligodendrocyte development in the optic nerve²⁰; and the development of Schwann cells in the peripheral nervous system.²¹

Mechanisms of apoptosis

All metazoan cells, from the early blastocyst to terminally differentiated neurons, will undergo apoptosis if deprived of survival factors, suggesting that death not survival is the default fate.²² It follows that the apoptotic machinery must be permanently in place and poised to act. Consistent with this, in many cases apoptosis can proceed in the absence of new gene transcription or protein synthesis.

The apoptotic programme can be broadly divided into three major stages: initiation; commitment; and execution (fig 1). While the

initiation step can be triggered by multiple stimuli, the commitment and execution steps appear to be more stereotyped.

Apoptosis can be initiated by diverse signals, including the withdrawal of survival signals, exposure to pro-apoptotic stimuli such as DNA damage, reduced intracellular ATP concentrations, or binding of ligands to specific receptors in the plasma membrane. Several such "death" receptors have been defined, including p75, Fas/APO-1, and the tumour necrosis factor (TNF) receptor.²³ Signal transduction from these receptors is mediated through a growing family of adaptor molecules that either directly or indirectly activate the enzymes that begin the dismantling of cell proteins and nucleic acids. In the case of Fas/APO-1, ligand binding induces receptor oligomerisation and subsequent binding of the Fas associating death domain protein, FADD. This molecule in turn recruits FLICE (FADD-like ICE), a member of the caspase family of proteases involved in orchestrating the apoptotic programme.²³

After the initiation of the apoptotic programme commitment to apoptosis is not immediate. The time at which a cell becomes irreversibly committed to death can vary from a few minutes to several hours or longer.²⁴ The decision to commit is affected by a delicate balance between pro- and anti-apoptotic forces within the cell. Many of the participants in the apoptotic cascade have opposing partners, so that the decision to live or die depends on whether pro- or anti-apoptotic signals are dominant. A growing number of second messengers have also been implicated in regulating the apoptotic process. These include protein kinase C cyclic AMP, calcium, and ceramide.²⁵ Until commitment is made, the outcome can be influenced by manipulation of these forces, and the process is subject to regulation. However, commitment marks a point of no return when anti-apoptotic intervention ceases to be effective.²⁶

The opposing aspects of cell survival and death are illustrated by the bcl-2 family of proteins. These genes are homologous to the anti-apoptotic gene, ced-9, identified in the nematode *C elegans* as an upstream inhibitor of the pro-apoptotic genes ced-3 and ced-4. In mammalian cells the bcl-2 family includes both anti-apoptotic proteins, such as bcl-x_i and bcl-2 itself, and their pro-apoptotic partners, including bax and bad.²⁷ Bcl-2 family members can form heterodimers with each other and it is the antagonistic balance between bcl-2 / bcl-x_i and bax / bad which controls the cellular decision to live or commit suicide. Bcl-2 was originally identified in chromosomal translocations in follicular lymphoma,²⁸ and this family of proteins also has a role in neuronal apoptosis. Although bcl-2 deletion in transgenic mice is not lethal and has no neuronal phenotype at birth,²⁹ substantial degeneration of motor, sensory, and sympathetic neurons occurs after the physiological cell death period. This suggests that bcl-2 is not a neurodevelopmental survival signal but is crucial for the maintenance of specific populations of neurons during the early postnatal period. In accordance with this, bcl-2

overexpression has been shown to protect mice against ischaemic stroke.³⁰ On the other hand, bcl-x₁ null mice die on embryonic day 13 with massive neuronal death in the brain and spinal cord, suggesting that this member of the bcl-2 superfamily is essential for normal neuronal development.³¹

One of the earliest signals in the apoptotic cascade is the activation of specific cysteine proteases that cleave at aspartate residues, now collectively called caspases.³² Caspase activation occurs by proteolytic cleavage of the pro-enzyme. For example, caspase-1 (also known as ced-3 or interleukin-1 β -converting enzyme (ICE)), is of primary importance because it activates at least two downstream caspases.³³ The caspases thus constitute an amplification mechanism for apoptotic signals. They are activated soon after the death trigger and cleave specific cellular targets, such as the DNA repair enzyme poly-ADP-ribose polymerase, presumably in preparation for ultimate cellular degradation. The importance of these enzymes in neuronal survival is illustrated by transgenic experiments where overexpression of a dominant negative caspase protects mice from cerebral ischaemia.³⁴

Recent work has emphasised the role of mitochondria in the regulation of apoptosis.³⁵ Bcl-2 family genes and ced-4 are located on the outer mitochondrial membrane, and genetic deletion of the membrane binding domains from the protein sequence attenuates their function. The release of cytochrome-c from mitochondria is required for execution to proceed in some cell lines, and mitochondria in apoptotic cells also release a pro-apoptotic peptide, apoptosis inducing factor. It has thus been suggested that the mitochondrion is the central control point of apoptotic execution, and that the opening of channels in the inner mitochondrial membrane, to cause the mitochondrial membrane permeability transition, is the final common pathway to apoptotic death. This hypothesis has several points in its favour, not least in offering an explanation of how cellular insults such as hypoxia-ischaemia can cause both apoptosis and necrosis.

In the late stages of apoptotic execution the organised degradation of genomic DNA is carried out by unidentified endonucleases. The activation of these enzymes is concurrent with, or immediately consequent on, caspase activation, although the precise mechanisms are as yet unknown. The end result of endonuclease activation is the internucleosomal cleavage of DNA, first leading to high molecular weight fragments (50 to 300 kb), and eventually to characteristic DNA "ladders" that can be detected by gel electrophoresis.³⁶

Methods for detecting apoptosis

As both apoptosis and necrosis can occur simultaneously, it has become increasingly important to distinguish between these two modes of cell death. Differences can be observed by light or electron microscopy, and no other method for distinguishing apoptosis from necrosis has been shown to be as reliable as morphological analysis using appropriate

stains. However, a variety of methods are in use, including: fluorescence activated cell sorting³⁷; detection of DNA fragmentation, either by agarose gel electrophoresis which demonstrates characteristic "ladders"³⁸; or the terminal deoxynucleotidyl transferase mediated biotin dUTP nick end-labelling (TUNEL) and in situ end labelling (ISEL) techniques which use DNA polymerase to repair DNA nicks with labelled nucleotides.³⁹

Apoptosis in nervous system injury and disease

Although apoptosis is involved in a number of diseases of the nervous system,⁴⁰ in most cases the link between a specific mutation or injury and the activation of apoptotic cascades remains elusive. A summary of a growing list of neurological diseases in which apoptosis has been implicated as an important pathological mechanism is given below.

NEURONAL INJURY

Cerebral hypoxic-ischaemic injury is a significant cause of death and neurological impairment. Magnetic resonance spectroscopy studies have shown that transient hypoxia-ischaemia leads to a biphasic disruption of cerebral energy metabolism. Related to this biphasic energy failure, two waves of cell death seem to follow hypoxic-ischaemic injury in the developing brain. Immediate neuronal death is probably largely due to necrosis, resulting mainly from the accumulation of calcium ions, leading to membrane pump failure.⁴¹ Delayed cell death resulting from hypoxic-ischaemic injury seems to involve different or additional mechanisms and a growing body of data suggests that in the delayed phase of injury cell death occurs by apoptosis.⁴²⁻⁴³ The amount of apoptosis is directly related to the magnitude of ATP depletion during hypoxia-ischaemia.⁴²⁻⁴⁴ Significant amounts of apoptosis have been observed in the brains of newborn infants following both birth asphyxia and sudden intrauterine death⁴⁵ and apoptosis is also prominent in white matter injury in newborn infants. There is evidence that apoptosis may continue for many weeks after an hypoxic-ischaemic insult,⁴⁶ and this may be related to the persistent changes in cerebral energy metabolism seen in infants during the months following birth asphyxia.⁴⁷

Following focal neural injury, apoptosis has been detected in sites distant from the initial damage. After acute spinal cord injury in monkeys apoptosis of oligodendrocytes occurs in remote degenerating fibre tracts,⁴⁸ and after forebrain injury in developing rats apoptosis can be observed in the cerebellum.⁴⁹ The apoptotic loss of oligodendrocytes could thus be a possible cause of secondary demyelination in paraplegia and in the chronic degeneration associated with multiple sclerosis. Further evidence for a role for apoptosis in this type of injury comes from the report that bcl-2 expression promotes the growth and regeneration of retinal axons.⁵⁰

NEURAL CANCERS

An intimate connection between apoptosis and the cell cycle has been shown in carcinogenesis: proto-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.⁵¹ For example, in a number of gliomas the loss of wild p53 activity has been linked to tumour progression, possibly resulting in resistance to chemotherapy and radiotherapy. Although there have been reports of *bcl-2* overexpression in glioma cell lines, the correlation between the anti-apoptotic effect of this gene and malignancy is not clear. However, a homologue of *bcl-2*, the brain related apoptosis gene (*BRAG-1*), is found predominantly in the brain, and is upregulated in human gliomas as a rearranged transcript.⁵²

INFECTIOUS DISEASE

Apoptosis may have a role in HIV encephalopathy. In the brain the virus replicates primarily in microglia which it enters via the CD4 receptor.⁵³ Although the activation of microglia is thought to be the primary cause of neuronal loss and demyelination, neurons die by apoptosis in HIV encephalopathies perhaps due to HIV mediated alterations in astrocyte function and aberrant stimulation of NMDA receptors, or by the accumulation of nitric oxide following the activation of inducible nitric oxide synthase.⁵³

In subacute sclerosing panencephalitis widespread apoptotic death has been detected in the brain,⁵⁴ although no correlation was observed between viral load, lymphocyte infiltration, and the number of apoptotic cells. DNA fragmentation indicative of apoptosis has been detected in scrapie infected sheep⁵⁵ and mouse⁵⁶ brain, suggesting a role for this mode of cell death in spongiform encephalopathies.

NEURODEGENERATION

Spinal muscular atrophy is associated with mutations in the survival of motor neuron and neuronal apoptosis inhibitory protein (NAIP) genes.⁵⁷⁻⁵⁸ NAIP is related to the baculovirus inhibitor of apoptosis protein and inhibits apoptosis in several cell types.⁵⁹ This implies that mutations in NAIP could deregulate apoptosis in spinal motor neurons, thus causing their death. The general importance of anti-apoptotic genes in neuronal protection is emphasised by recent reports that NAIP overexpression can also rescue neurons after cerebral ischaemia.

Apoptosis has also been implicated in retinal dystrophies such as retinitis pigmentosa. In this instance, apoptosis results from mutations in any one of the three photoreceptor genes rhodopsin, peripherin, and the β -subunit of cyclic guanosine monophosphate diesterase, resulting in photoreceptor degeneration.⁶⁰ Although the apoptotic trigger is unknown, absence of *c-fos* prevents apoptosis in these cells.⁶¹ Moreover, defined neurotrophins and growth factors injected intraocularly in animal models of retinal degeneration enhance photoreceptor survival, implying that the apoptotic

cascade can be blocked by supplying exogenous survival signals.⁶²

The mutation underlying Huntington's disease is an expanded trinucleotide repeat within a novel gene, huntingtin, that is crucial for normal development and can be regarded as a cell survival gene.⁶³ Transgenic models null for the huntingtin gene show increased apoptosis in neurons of the embryonic neuroectoderm.⁶⁴ Moreover, huntingtin is specifically cleaved during apoptosis by caspase-3 (apopain) and the rate of cleavage is enhanced by a gain of function associated with the triplet expansion.⁶⁵ This is also supported by the observation that overexpression of the specific trinucleotide repeats in transgenic mice is sufficient to induce the Huntington's phenotype.⁶⁶

Most cerebellar ataxias are associated with neuronal loss. Ataxia telangiectasia, caused by mutations in the ATM gene, is thought to have an apoptotic component. ATM shares extensive and important homology with the DNA dependent protein kinases involved in DNA damage responses at different cell cycle check points, and is downregulated in all patients with ataxia telangiectasia.⁶⁷ The fact that inappropriate p53 mediated apoptosis is the major cause of death in ataxia telangiectasia cells irradiated in culture⁶⁸ suggests that the mutation causes inappropriate triggering of apoptosis by otherwise non-lethal DNA damage.

In the familial form of amyotrophic lateral sclerosis gain of function mutations in the gene encoding copper zinc superoxide dismutase (*sod-1*) create a dominant pro-apoptotic signal.⁶⁹ Although cell injury by accumulation of free radicals can trigger apoptosis, these mutants can induce apoptosis both in neural cells in culture and in transgenic mice.⁷⁰⁻⁷¹

Mental retardation in Down's syndrome has also been linked to inappropriate apoptosis. Although cortical neurons from fetal Down's syndrome brains differentiate normally in culture, they subsequently degenerate and undergo apoptosis.⁷² Degeneration is blocked by treatment with free radical scavengers, suggesting that a defect in metabolism of reactive oxygen species is the trigger for apoptosis.

In Parkinson's disease the death of dopaminergic neurons in the substantia nigra has been shown to occur by apoptosis and can be blocked by delivery of glial derived neurotrophic factor.⁷³ Alzheimer's disease is associated with the progressive accumulation of β -amyloid protein which is the major component of neural plaques.⁷⁴ The β -amyloid peptide can induce neurons to undergo apoptosis in vitro.⁷⁵

INHERITED METABOLIC DISEASES

There are some data to suggest that the acute encephalopathy associated with maple syrup urine disease is due to the induction of apoptosis by an accumulating metabolite of leucine, α -keto isocaproic acid.⁷⁶ This compound is a potent inducer of apoptosis in central nervous system glial cells in culture, and the effect is enhanced in the presence of leucine. Phenylalanine and leucine alone do not induce

apoptosis in this system, suggesting that this effect is specific (unpublished observations).

Therapeutic manipulation of the apoptotic programme

INHIBITION OF APOPTOSIS

As apoptosis is a highly regulated physiological process, a window of opportunity exists for therapeutic intervention. However, if anti-apoptotic strategies are to be successful, first it must be established that apoptosis is a major component leading to the neuronal deficit, second that inhibition of cell death does not result in the persistence of damaged neurons, and third, that the anti-apoptotic treatment can be applied before the cellular commitment to apoptosis.

Several anti-apoptotic agents reduce neuronal loss following cerebral ischaemia. Both IGF-I⁷⁷ and the protein synthesis inhibitor cycloheximide⁷⁸ reduce cerebral injury if administered soon after hypoxia-ischaemia. Similarly, administration of a peptide inhibitor of caspase activity (z-VAD-DCB) substantially reduces infarct volume resulting from focal cerebral ischaemia in rats.⁷⁹ Our own studies have shown that mild hypothermia applied immediately after cerebral hypoxia-ischaemia prevents neural apoptosis without affecting the amount of necrosis.⁸⁰ Inhibitors of neuronal apoptosis also have therapeutic value in neural trauma. Neurotrophins directly introduced into central nervous system lesions rescue degenerating neurons,⁸¹ suggesting a possible therapeutic intervention for neurodegeneration and paraplegia. In this respect, targeted delivery of normal alleles to replace mutated genes or anti-apoptotic genes, to enhance survival of degenerating neurons, is the long term goal of gene therapy.⁸²

INDUCTION OF APOPTOSIS

The anti-tumour effect of radiotherapy and chemotherapy is effected through the induction of apoptosis in tumour cells. However, the considerable side effects of these pro-apoptotic treatments have led to the search for novel methods for promoting tumour apoptosis. Brain tumours have been treated successfully using the herpes thymidine kinase-gancyclovir gene therapy approach which works by targeting dividing cells in a non-proliferating environment⁸³; several clinical trials are now underway in humans. As gliomas respond well to Fas/Apo-1 mediated induction of apoptosis⁸⁴ this could also lead to the development of a therapeutic intervention.

In summary, there is accumulating evidence for the role of neuronal apoptosis in diseases of the nervous system. Researchers are now looking forward to the exciting prospect of developing effective therapeutic strategies based on the manipulation of this physiological process.

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