Ca²⁺ signals and death programmes in neurons

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Cell death programmes are generally defined by biochemical/genetic routines that are linked to their execution and by the appearance of more or less typical morphological features. However, in pathological settings death signals may engage complex and interacting lethal pathways, some of which are common to different cells, whereas others are linked to a specific tissue and differentiation pattern. In neurons, death programmes can be spatially and temporally segregated. Most importantly physiological Ca²⁺ signals are essential for cell function and survival. On the other hand, Ca²⁺ overload or perturbations of intracellular Ca²⁺ compartmentalization can activate or enhance mechanisms leading to cell death. An imbalance between Ca²⁺ influx and efflux from cells is the initial signal leading to Ca²⁺ overload and death of ischaemic neurons or cardiomyocytes. Alterations of intracellular Ca²⁺ storage can integrate with death signals that do not initially require Ca²⁺, to promote processing of cellular components and death by apoptosis or necrosis. Finally, Ca²⁺ can directly activate catabolic enzymes such as proteases, phospholipases and nucleases that directly cause cell demise and tissue damage.

Keywords: calcium; neurodegeneration; apoptosis; excitotoxicity

1. INTRODUCTION

It is well established that genetically encoded programmes decide the fate of individual cells or organs during development and in normal tissue cell turnover. However, in recent years it has also become clear that cells execute one or more biochemical programmes to signal or execute cell death also under pathological conditions. In many instances, developmental cell death and death under pathological conditions share similar morphological features as well as signals and execution systems. For example, the characterization of the main signals, modulators and executioners of programmed cell death in *Caenorhabditis elegans* has led to the understanding of very important death pathways in pathological cell death of mammalian organisms. This is not a singularity of the death programme that we call apoptosis, but the concept can be extended to other paradigms of cell death. For example, autophagic cell death, whose main feature is the presence of cytoplasmic lysosome-derived vacuoles, is frequent in both neuronal development and neurodegenerative disease (Yuan *et al.* 2003). The existence of conserved biochemical pathways to signal and execute cell death has somewhat reduced the emphasis on the morphological characterization of cell death. Despite the efforts to define individual forms of cell death based on their appearance, it is clear that cell disassembly in all circumstances involves nuclear fragmentation/dissolution, organelle disruption (sooner or later) and eventually membrane lysis and phagocytosis. Thus, condensation and eventual fragmentation of the nucleus occurs in apoptosis as well as in necrosis and autophagic cell death. Both in apoptosis

and necrosis, the mitochondria can be partially or totally damaged and again the cytoskeletal structure is compromised and molecules are exposed at the cell surface to promote recognition and scavenging. Therefore, the concept of a death programme is not necessarily linked to a morphological appearance. There is increasing evidence that apoptotic-like features can also be found when the main executioners of apoptosis, the caspases, are inhibited (Volbracht *et al.* 2001), whereas caspase-mediated cleavage of relevant substrates may be involved in cell lysis/necrosis (Schwab *et al.* 2002). Also, under pathological conditions, apoptotic and non-apoptotic death paradigms are often intertwined, thus suggesting that, *in vivo*, cells may use concomitantly diverging execution pathways. Thus, under pathological conditions several protease families may cooperate to disassemble cells by targeting different organelles or cellular substructures. For example, it is remarkable that caspases and calpains share many substrates. Notably the cleavage sites for the two protease families are in several cases located on very close regions within the same protein (Schwab *et al.* 2002). While the predominance of one or another death executing mechanism may be dictated by factors as different as energy requirement, signalling molecules and the intensity of a given insult, in many instances the differentiation programme within a given cell tissue dictates the way to die.

For example, growing evidence suggests that synapse, projections and soma represent distinct degenerative compartments in a neuron. Several recent observations suggest the existence of death programmes, which are precisely controlled spatially and temporally. Interestingly, the same machineries/players implicated in mechanisms of cell death seem to be also involved in the physiological dismantling of neuronal

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structures such as synapses and axons. Thus, forms of axon pruning in development seem to share similarities with degeneration of axons in response to injury (Watts *et al.* 2003).

Also tissue-specific signalling systems such as the *N*-methyl-D-aspartate receptor (NMDA-R) in neurons may dictate whether, and how, a neuron dies. Early studies have shown that over-stimulation of NMDA-R can cause either apoptosis or necrosis depending on the intensity of the insult (Bonfoco *et al.* 1995b). Subsequent work has shown that synaptic glutamate stimulation may predominantly elicit apoptosis (Leist *et al.* 1997), whereas excess glutamate causes predominantly necrosis (Bano *et al.* 2005). Here, we describe some of the latest insights on the compartmentalization of degenerative processes in neurons and on the death mechanisms triggered by NMDA-R activation in neurons.

2. SPATIALLY AND TEMPORALLY DISTINCT PROGRAMMES FOR NEURONAL DEATH

Since the first description of axonal degeneration by Waller (1850), the mechanisms underlying the degradation of an injured axon have received considerable attention particularly in the peripheral nervous system (Schaumburg *et al.* 1974; Coleman & Perry 2002). However, the degeneration of the distal part of an axon, occurring in peripheral neuropathies or following an injury, was considered to be a passive process until the characterization of the Wld^s mouse (Lunn *et al.* 1989; Sievers *et al.* 2003). This natural mutant, characterized by a remarkably slow axonal degeneration, opened the door to the hypothesis that axonal disintegration can be an autonomous and active process. Compelling evidence suggests now that different execution systems operate in the axons and in the cell bodies during Wallerian degeneration (Finn *et al.* 2000; Raff *et al.* 2002). Caspase-independent axonal disintegration associated with caspase-dependent apoptosis of the cell body has been described in Wallerian degeneration of optic and sciatic nerve explants and in localized axonal dismantling induced by local neurotrophin deprivation in dorsal root ganglia (Finn *et al.* 2000). However, apoptotic features such as phosphatidylserine exposure and loss of mitochondrial membrane potential have been reported in other models of Wallerian degeneration independently of caspase activation (Sievers *et al.* 2003). The observation that in nerve growth factor-deprived sympathetic neurons, proteasome inhibitors are able to delay the degeneration of the neurites, but not the apoptosis of the cell bodies, provides additional support to the existence of distinct programmes operating during the dismantling of the neuronal projections (Zhai *et al.* 2003).

Only in more recent years, the concept of localized axonal degeneration and dying-back processes have been applied also to the central nervous system (CNS). Disruption of synapse integrity or function has been suggested to be even more relevant than neuronal loss in slow central degenerative disorders such as Alzheimer (AD), Huntington (HD) and Parkinson's diseases. Loss of synapses appeared proportionally greater than the loss of neurons in AD patients (Davies *et al.* 1987),

and a good correlation has been reported between cognitive decline and loss of pre-synaptic terminals (Sze *et al.* 1997; Hatanpaa *et al.* 1999). A marked decrease in striatal volume, without changes in cell number, has also been observed in mouse models of HD (Mangiarini *et al.* 1996; Yamamoto *et al.* 2000), thus suggesting that loss of neurites and synaptic failure may determine the HD phenotype.

Since localized axonal degeneration or dying back processes can be difficult to investigate *in vivo*, particularly in the CNS, cellular models have been developed that allow dissection of the main signals involved in the dismantling of the different cellular compartments. The mechanical cut of the axon and the local deprivation of growth factors are two classical *in vitro* systems (see Raff *et al.* 2002). However, axonal degeneration and neuronal cell death can also be induced chemically by the microtubule-interfering agent colchicine (Bonfoco *et al.* 1995a; Volbracht *et al.* 1999) or, as shown recently, by interfering with synaptic function with botulinum neurotoxin C (BoNT/C) in central neurons (Berliocchi *et al.* 2005). In this case, the widespread but selective synaptic damage elicited by BoNT/C initiates an early degeneration of the neurites independent of caspase and characterized by the presence of functional mitochondria. On the contrary, loss of mitochondrial membrane potential, cytochrome *c* release and caspase activation accompany the late demise of the cell bodies. Although caspase-independent mechanisms mediate the initial neurodegenerative events, the presence of processed caspase-3 along the projections of BoNT/C-treated neurons suggests either the existence of very effective anti-apoptotic machinery or an undefined house keeping function for this family of proteases. Growing evidence suggests that many of the same biochemical and molecular players involved in the death of the cell bodies are also engaged in the localized dismantling of synaptic terminals and neurites in physiological conditions. Thus, processed caspase-3 has been detected in retinal growth cones. Here its activation, confined to a specific compartment, does not trigger the full apoptotic cascade but rather results in transient, localized changes in specific proteins involved in cone collapse and chemotropic turning (Campbell & Holt 2003). In a similar way, the ubiquitin-proteasome has been implicated in apoptosis (Sun *et al.* 2004) axonal degeneration (Zhai *et al.* 2003), as well as in neurodegenerative diseases. However, the same system is required for physiological axonal pruning (Watts *et al.* 2003).

This suggests that the cell death linked pathways: (i) may be used locally to eliminate unnecessary or injured structures and (ii) may have a physiological function, independent of their role in cell death. Therefore, the activation of local apoptotic processes in differentiated neurons is not detrimental *per se*, but it may be required as a physiological and important response.

3. DISTINCT DEATH ROUTINES IN BRAIN ISCHAEMIA: THE ROLE OF Ca^{2+} SIGNALS

The diverse actions of intracellular Ca^{2+} signals provide an optimum example of the fact that the

same signal may be physiological or detrimental depending on threshold and cellular conditions. Tight homeostatic mechanisms regulate intracellular Ca^{2+} concentration in order to retain Ca^{2+} signals spatially and temporally localized (Criddle *et al.* 2004) and to allow multiple Ca^{2+} -mediated signalling cascades to occur independently within the same cell. However, excessive Ca^{2+} influx, release from intracellular stores or impairment in the Ca^{2+} -extruding machinery, can overcome Ca^{2+} -regulatory mechanisms and lead to cell death (Arundine & Tymianski 2003; Orrenius *et al.* 2003).

Several lines of evidence suggest the key role of Ca^{2+} in excitotoxicity induced by glutamate (Arundine & Tymianski 2003); however, the cellular subroutines engaged in excitotoxic cell death are still debated. Thus, depending on the extent and the duration of the Ca^{2+} influx, neurons will survive, die by apoptosis (i.e. sustained slow Ca^{2+} influx), or undergo necrotic lysis (i.e. rapid high Ca^{2+} influx; Ankarcrona *et al.* 1995; Choi 1995).

Ca^{2+} signals can trigger cell death or reinforce the execution of death subroutines (Orrenius *et al.* 2003). Redistribution of Ca^{2+} within intracellular stores can amplify apoptotic signals (Scorrano *et al.* 2003), but can also initiate cell death execution by calpains (Nicotera *et al.* 1986). To keep Ca^{2+} fluctuations within physiological levels and avoid Ca^{2+} overload cells have developed very efficient systems. The major long-term regulators of the intracellular Ca^{2+} content are the plasma membrane (PM) Ca^{2+} -extrusion systems. In neurons, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) has the highest capacity, whereas the ATP-driven plasma membrane Ca^{2+} pump (PMCA) has a high affinity for Ca^{2+} but a low turnover number (Carafoli *et al.* 2001).

Regardless of the source of the calcium overload, the integrity of the PM extruding systems is essential to restore physiological intracellular Ca^{2+} levels. Even in cells where the apoptotic death routine has been engaged, the impairment of this machinery can trigger other lethal mechanisms (Schwab *et al.* 2002; Bano *et al.* 2005). In a model of neonatal hypoxia–ischaemia, and in cultured neurons, where excitotoxicity is triggered by synaptic glutamate release, caspases can cleave the PMCA causing a loss of function, aberrant intracellular Ca^{2+} transients, and a Ca^{2+} overload, which finally results in cell lysis. It is interesting to note that synaptic glutamate release causes predominantly an apoptotic-like death routine (Bonfoco *et al.* 1996; Leist *et al.* 1998) with caspase activation. This shows that caspases can also be involved in a typical necrotic death routine (i.e. Ca^{2+} overload leading to cell lysis). These findings also suggest an explanation for the protective effect of caspase inhibitors on secondary necrosis in stroke models, where ischaemic areas which are clearly necrotic can be rescued if caspases are inhibited (Hara *et al.* 1997a,b). The hypothesis that the cleavage of PM Ca^{2+} transporters is a relevant subroutine of the neuronal death programme has been further supported by more recent data. During brain ischaemia and in neurons exposed to excitotoxins the NCX, and in particular the isoform 3, is cleaved and inactivated. However, in this case, calpains

mediate NCX degradation. Over-expression of the endogenous calpain inhibitor, calpastatin, or the calpain-resistant isoform NCX2 prevent Ca^{2+} overload and protect neurons (Bano *et al.* 2005).

4. CONCLUSION

It is not surprising that initially simple death programmes, originated early during phylogeny, undergo complex modifications in mammalian cells. Large gene families have evolved to provide a more intricate control of cell death in higher organisms, in part perhaps to accommodate the need of individual organ differentiation. Some characteristics of the original cell death machinery that would affect predominantly the shape of death may have become more significant or predominant in some subsets of mammalian cells. A further consequence of the increased complexity may be that an increasing number of feed-back loops gives rise to multiple possibilities of initiation, control and execution.

Stimulation of self-feeding death subroutines, which maintain both the activation of executioners and the neutralization of defence systems, is necessary for the completion of most death programmes. The main implication of this standpoint is the exclusion of a single, predominant and molecular-defined commitment step. It seems likely that accumulation of damage incompatible with cell survival would require disruption of several vital functions. Once such a threshold is trespassed, multiple positive feed-back loops would ensure the progression of the death programme to the end, and the safe disposal of the injured cell. This also implies that the morphological appearance of cell death is not linked to a single commitment point, but rather is the result of a more or less complete execution of subroutines deciding on the shape of dying cells.

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