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DANCE ROUND

WE DANCE ROUND *IN A RING AND SUPPOSE,*
BUT THE SECRET SITS IN THE MIDDLE AND KNOWS.
ROBERT FROST

APOPTOSIS IN NEURODEGENERATIVE DISEASES: TO BE OR NOT TO BE?

ABSENCE OF PROOF IS NOT PROOF OF ABSENCE

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• Apoptosis (from Greek - 'falling off) is a term coined by Kerr, Wyllie and Currie in 1972 to describe a form of cell death associated with peculiar morphological changes (1). They contrasted apoptosis with necrosis, in which large numbers of cells undergo destruction and elicit a regional inflammatory response. In contrast, in apoptosis individual cells die and are being removed quickly, without inflammation, making their demise often difficult to detect. The initial concept of apoptosis was exclusively related to a morphological phenomenon.

Interest surged in the mid-eighties, when it became clear that this morphologically defined form of cell death indicated a controlled form of cell demise associated with a large number of physiological and pathological processes. Apoptotis soon became recognized as playing a role in a multitude of eukaryote biological processes, such as steady-state cell turnover in healthy tissues, insect metamorphosis or the regulation of the cell number in the nervous and immune system. In addition, apoptosis occurred in pathological states, after growth factor or hormone deprivation, drug exposure, ionizing radiation, hypoxia, overexpression of various viral and cellular proteins, and in neoplasms (2). For processes of excess cell removal during growth and development the term programmed cell death (PCD) was introduced and it became associated and in fact loosly equated with apoptosis. Quite significantly, PCD can be inhibited by RNA transcription inhibitors like actinomycin-D, or protein synthesis inhibitors like cycloheximide. Thus, PCD/apoptosis is an actively regulated process that requires the expression of various genes in order to take place.

The third stage was set when the molecular mechanisms underlying controled cell death were unraveled in cell culture systems, simple organisms like *C. elegans,* or organotypic preparations (3,4). Both PCD and the various traumatic stimuli mentioned turned out to share similar molecular pathways, all leading to that final common pathway of morphological changes initially called apoptosis. Moreover, a surprising conservation of genes involved was found throughout eukaryotic cells, as well as in viruses.

In the developing nervous system, PCD and apoptotic removal of excess neurons constitute an important mechanism responsible for the mature architectural configuration (5). It has been postulated that apoptosis is also the mode of neuronal destruction in human neurodegenerative diseases (3,6). Although circumstantial evidence may seem to support this view, in this *Dance Round* we will try to demonstrate that up to now nobody has unequivocally demonstrated apoptosis in the nervous system of patients who died with neurodegenerative diseases. No one has been able to fulfill the strict morphological and molecular criteria for apoptosis in human postmortem brain. But absence of proof is not proof of absence: rather than due to non-existence, the difficulties of demonstrating apoptosis may be a result of the peculiar characteristics of the process.

• Apoptosis: morphological criteria and molecular mechanisms

Apoptosis involves a highly characteristic sequence of mor-

phological alterations. In the earliest phase, cells undergo aggregation of nuclear chromatin into large, compact masses localized at the membrane and resulting in an irregularly convoluted nuclear surface. Somewhat later the nucleus becomes indented and ultimately fragmented. This process is best observed by transmission electron microscopy but may be revealed in light microscopy as pyknosis and karyorrhexis, respectively. At the same time cytoplasmic condensation takes place. Cells shrink, organelles crowd, but do not lose their integrity, and become sequestered within membraneous envelopes that initially form blebs on the cell surface, soon to be detached as apoptotic bodies. These bodies, as well as the remains of the original cell, become engulfed in tissue resident cells or in macrophages, without inflammatory response elicited. Characteristically, it may take only a few hours before a cell undergoing apoptosis is completely removed, although intracellular apoptotic bodies that have been scavenged may be detectable for more than 12 hours (2). Fig. 1 shows apoptotic cells in a classical model, the rat prostate 3 days after castration.

Of the various molecular events associated with apoptosis, some important ones are related to DNA degradation. In rodent thymocytes undergoing apoptosis after treatment with glucocorticoids, a characteristic "DNA-laddering" can be found. Electrophoretic separation of extracted DNA on agarose gels reveals fragments that are multiples of-180 bp. This periodicity is due to multiple single-strand nicks in the internucleosomal linker region and follows the initial generation of much larger fragments associated with the unfolding of chromatin loop domains (7). The endonucleases considered to be responsible for internucleosomal cleavage include DNase I, DNase II, and NUC 18, an 18 kD DNase isolated from glucocorticoid-treated thymocyte nuclei (7). Methods have been developed that use *in situ* end-labeling (ISEL) to demonstrate in individual cells the existence of DNA breaks. *In situ* nick translantion (ISNT) employs DNA polymerase I, while TdT-mediated dUTP nick-end labeling (TUNEL) employs terminal deoxunucleotidyl transferase (8-10).

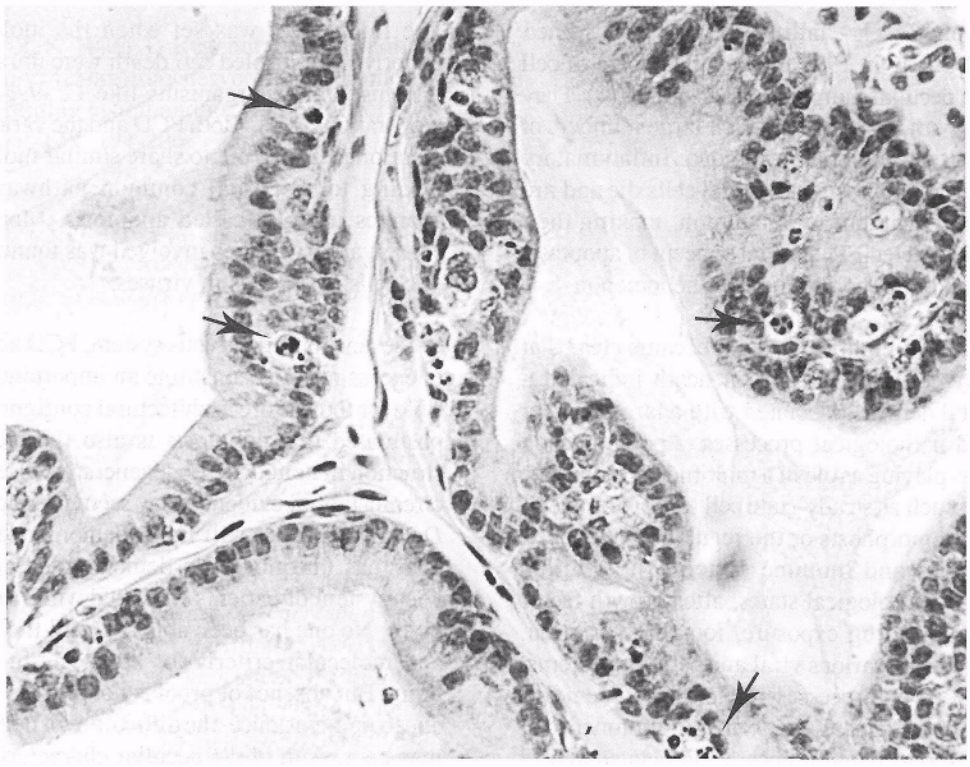

Figure 1. *Cells undergoing apoptosis in a classical model, the rat prostate 3 days after castration. Arrows point to apoptotic cells. Hematoxylin and eosin.* x *40.*

Internucleosomal cleavage, chromatin condensation and membrane redistribution are the final steps in an extensive molecular pathway that involves an intricate machinery of apoptosis inducing and inhibiting factors. The most extensively studied proteins participating in this machinery are proteins from the *bcl-2* family. *Bcl-2* is a proto-oncogen that was originally identified in B cell lymphomas at a chromosomal t(14;18) breakpoint that juxtaposes the chromosome 18 *bcl-2* gene to the immunoglobulin heavy chain locus on chromosome 14. Thus, a *bcl-2-lGR* fusion gene is created that is overexpressed in lymphoid tissue and results in a polyclonal expansion of small resting B cells that fail to die (11-13). These findings implied that *bcl-2* is an apoptosis-inhibiting gene. Consistent with this notion, *bcl-2* showed a surprising 23% homology to a *C. elegans* death-protecting gene, *ced-9,* and could in fact rescue *ced-9* mutants from untimely apoptotic death (14). A protective role for the *bcl-2* protein in apoptosis has now been demonstrated in a variety of experimental systems, including cell death induced by nerve growth factor (NGF) withdrawal, gamma irradiation, and cancer chemotherapeutics (15). In addition, the protein plays a physiological role in B-cell and T-cell development. As demonstrated by immunohistochemistry, *bcl-2* is expressed in a wide variety of tissues, including germinal centers of lymphoid follicles, precursor cells of hematopoietic lines, various glandular and epithelial tissues, particularly hormone-dependent forms, and neurons in the temporal cortex (16). No immunorectivity was found in glial cells.

Various *bcl-2* -related genes have been identified that may also be involved in apoptosis regulation. These genes include *bcl-x* (with its two splice variants, a large one, *bcl-x*$_l$ and a small one, *bcl-x), MCL1,* and *box.* This group of genes shares at least two homologous domains, called BH1 and BH2 (17). Like *bcl-2,* the *box* protein is widely distributed in various cell types. In the central nervous system, *bax* immunorectivity was localized in spinal motor neurons (particularly if they showed degenerative features), dorsal ganglion sensory neurons, brainstem motor neurons and reticular neurons, Purkinje cells, and cortical large and medium sized neurons (18). Again, *bax* immunoreactivity was absent in glial cells and Schwann cells. It was noted that *bax* and *bcl-2* immunoreactivity do not to overlap completely (18). This finding is interesting as *bax* and *bcl-2* seem to excercize opposing roles in apoptosis regulation. Whereas *bcl-2* prevents cell death, *bax* expression is associated with the occurrence of apoptosis. The two proteins are able to form homo- or heterodimers, a proces dependent upon the integrity of the BH1 and BH2 domains (19). The relative amounts *ofbcl-2* and *bax* ultimately determine a cell's fate: with excess *bcl-2, bcl-2* homodimers are formed predominantly, resulting in cell survival, whereas excess *bax* results in *bax* homodimers and apoptosis (20). The presence of *bcl-2/bax* heterodimers may thus represent a finely balanced intermediate state.

A host of other gene products contribute to this system of checks and balances. Additional death promoting factors that have been identified include *bcl-x*$_l$ and interleukin converting enzyme (ICE; homologous to the *C. elegans* death promoting gene *ced-3).* In contrast, *bcl-x* $_l$ and *crm* A, a cowpox virus gene, prevent cell death. *Bcl-x*$_s$ and *bcl-x* $_l$ may form a complementary pair similar to *bcl-2* and *bax.* Moreover, extensive crosstalk seems possible. Both *crmA* and *bcl-2* are able to repress ICE-dependent apoptosis in NGF-depleted cultured dorsal root ganglion cells, without additive or synergistic effects, suggesting a mode of action that involves one site (21). Currently, these complicated cellular networks that regulate apoptosis are only incompletely understood, but they may be crucial for our understanding of neurodegenerative diseases,

- **Apoptosis in neurodegenerative disease**

Due to the non-inflammatory nature of neuronal falling off in neurodegenerative diseases, and the regional selectivity of degeneration in most of these diseases, apoptosis has been considered to be the mechanism of cell death in diseases such as Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), or Huntington disease (HD). However, demonstrating apoptosis in human postmortem brain is difficult, primarily due to the kinetics of the process. An apoptotic neuron is no longer visible after a few hours. In contrast, neurodegenerative diseases typically take many years to develop. Therefore, one can not expect to find more than a few neurons at most in a postmortem brain. Careful, exhausting quantitation of apoptotic neurons will be required to demonstrate the presence of apoptosis in affected brains unequivocally.

Various studies have reported evidence of extensive ISEL/TUNEL DNA-labeling in AD and HD (22-25), although dissenting opinions can be found (10). One recent study reported finding up to 10 % of TUNEL labeling neurons locally in the striatum of HD brains (24). This finding could only be explained by assuming that neurodegeneration in this disease is a process that is locally restricted in time, a notion clearly not in line with the clinical progress of the disease. Although the presence of ISEL/TUNEL-positive neurons as often considered proof of apoptosis in the brains under consideration, the specificity of the method remains to be established, particularly in the postmortem central nervous system. In our experience, TUNEL or ISEL in postmortem human central nervous system tissue labels far too many nuclei, probably through random DNA breaks (Fig. 2). This makes it difficult to distinguish the few apoptotic neurons from the vast majority of neurons with necrotic, agonal, or postmortem DNA damage. TUNEL/ISEL labeling has been demonstrated quite convincingly in ischemia models (26,27).

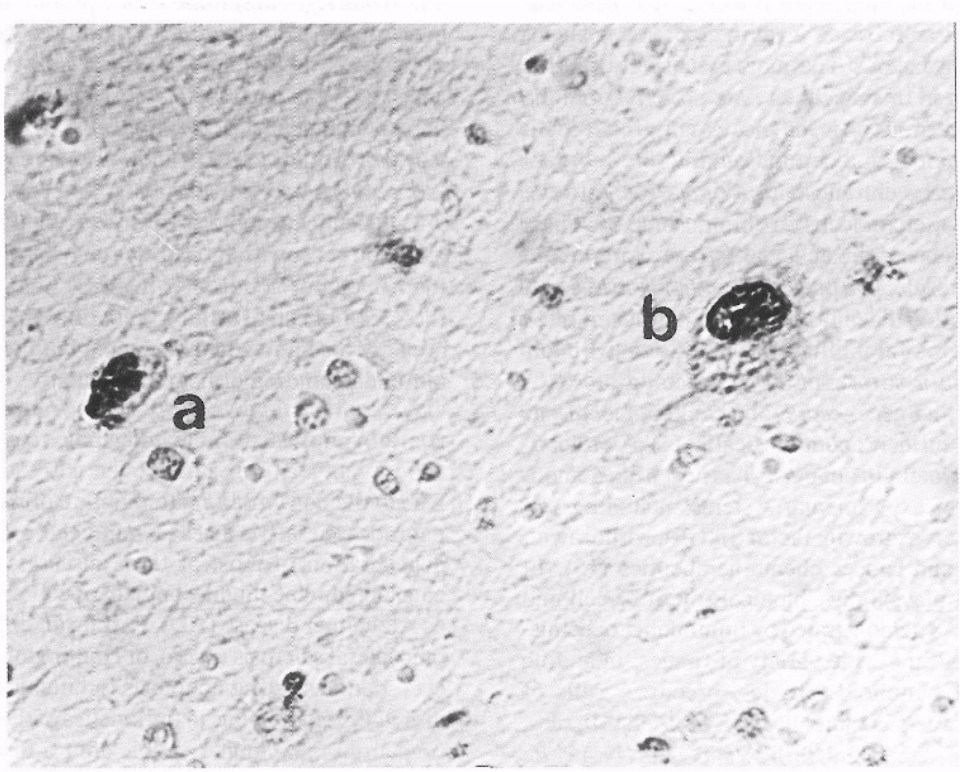

***Figure** 2. TUN EL labeled neurons in the striatum oj'Huntington's disease patient, (a) Nuclear morphology suggestive of apoptosis. **(b)** Morphology suggestive of random DNA labeling. x400.*

Circumstantial evidence has been derived from studies in cultured cells. A role for PCD in human ALS was suggested by an apoptosis occurring in motoneurons deprived of trophic support (28), or in spinal neurons after chronic inhibition of superoxide dismutase (29). Apoptosis occurred in PC-12 pheochromocytoma cells treated with complex I inhibitors (30,31), and in ventral mesencephalic neuronal cultures after treatment with MPP+ (32), providing a link to PD. *Ed-2* transfected and expressing PC-12 cells were protected against dopamine toxicity (33). Beta-amyloid may induce apoptosis in cultured cortical neurons (34). Although these results are suggestive to some extent of apoptosis playing a role, a direct extrapolation to the relevant human neurodegenerative disease is tenuous at best.

If, either in human brains or animal models, alterations in the expression of proteins like *bcl-2, bax,* ICE or others will be demonstrated, that would constitute stronger evidence in favour of apoptosis. However, the most compelling evidence for a role of apoptosis would come from the elucidation of the function of mutated genes or altered proteins. A recent study reported increased apoptosis in embryonic mice with a nullizygous knock out of the HD gene, suggesting, but not proving, a regulatory role for this gene in apoptosis (35). But currently the best case may be made for autosomal dominant spinal muscle atrophy (SMA). In line with an older study demonstrating apoptotic muscle fibers in a fulminant form of childhood SMA (36), a recent study found deletions in a gene with remarkable homology to the baculoviral *cp-* and *op*-IAP anti-apoptosis genes (37). Clearly, this now constitutes the best available evidence for any neurodegenerative disease of a role for apoptosis. Still, this mutation is not unambiguously established (38,39).

In conclusion, the process of controled cell death, apoptosis, seems crucial to our understanding of normal cell physiology. In addition, it plays a role in pathologial cell death, and it has been proposed to take place in various human neurodegenerative diseases. However, the currently available evidence seems insufficient to establish beyond doubt the existence of apoptosis in any neurodegenerative disease. Methodological considerations still complicate the search for apoptotic changes

in human postmortem material, and novel approaches are needed. One could argue that, on its own, the demonstration of apoptosis is not very relevant: the underlying molecular mechanisms need to be unraveled. But a demonstration of apoptosis would imply a restricted set of molecular pathways, focus further research efforts, and open up therapeutic strategies. It would embed research into these diseases into a far broader field of cellular biology: clearly a beneficial development for patients and their families.

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