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A First Insight into the Molecular Mechanisms of Apoptosis

Commentary

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Our 1993 paper entitled "The *C. elegans* Cell Death Gene *ced-3* Encodes a Protein Similar to Mammalian Interleukin-1-β-Converting Enzyme" (Yuan et al., 1993) announced the discovery of a molecular mechanism for programmed cell death in the nematode *Caenorhabditis elegans* and led us to suggest that this mechanism is likely conserved in mammals. Our first glimpse of the impact this paper would have on the fields of biology and medicine came the day this paper was published: that day a number of pharmaceutical companies contacted one of us (H.R.H.) to ask how this finding could help them discover drugs. Nonetheless, its subsequent effect on the field of mammalian apoptosis was beyond our imagination at the time.

In the past decade, the field of apoptosis has become enormous, with more than 10,000 published papers directed toward an understanding of CED-3-like proteases, now known as caspases, and more than 70,000 papers toward the understanding of apoptosis in general. Many of these latter papers use caspase activation as their primary marker for the process of apoptosis. The Yuan et al. (1993) paper, coupled with the companion paper by Miura et al. (1993), which showed that CED-3 expressed in mammalian cells could induce those cells to undergo apoptosis, not only provided the first insight concerning the molecular mechanisms of apoptosis but also established one of the first two (also see Vaux et al., 1992) direct links between the genetic analysis of programmed cell death in *C. elegans* and the field of mammalian apoptosis.

That *ced-3*, a gene required for programmed cell death in *C. elegans*, encodes a protein resembling mammalian interleukin-1β converting enzyme, a cysteine protease now termed caspase-1, strongly suggested that the CED-3 protein functions as a cysteine protease in regulating programmed cell death in worms. *ced-3* had been identified by Hilary Ellis in a genetic screen for mutations that suppress the presence of persistent cell corpses caused by a mutation in *ced-1*, a gene required for the engulfment of dying cells (Ellis and Horvitz, 1986). In wild-type worms, cells undergoing programmed cell death are efficiently recognized, engulfed, and degraded by neighboring cells. The engulfment process is now known to be regulated by at least seven genes, which encode components of two parallel and partially redundant signal transduction pathways (e.g., Reddien and Horvitz, 2000; Zhou et al., 2001). *ced-1*, the first engulfment gene to be identified, was discovered by Ed Hedgecock in a genetic screen for morphological mutants using Nomarski microscopy (Hedgecock et al., 1983). The engulfment defect of *ced-1* delays the degradation of dead cells and provided a genetic background that allowed the ready visualization of accumulated unengulfed cell corpses.

The first *ced-3* mutation was isolated because it eliminated the persistent cell corpses observed in *ced-1* animals. Further analysis revealed these corpses were absent because without *ced-3* function programmed cell death was not initiated. This observation demonstrated that *ced-3* acts as a killer gene in programmed cell death. Although the ability of inhibitors of protein and RNA synthesis to block metamorphic cell death in tadpoles and insects and to block certain cell deaths in culture had led to suggestions that cell death required gene activity (e.g., Lockshin, 1969; Tata, 1966), it was the discovery of *ced-3* (and of the second *C. elegans* killer gene *ced-4*; Ellis and Horvitz, 1986) that directly demonstrated the existence of genes that act in the process of programmed cell death.

How *ced-3* might regulate programmed cell death was a frequent subject of debate in our laboratory at the time. Since an increase in levels of the hormone 20-hydroxyecdysone had been shown to be critical in determining the onset of metamorphic cell death in insects (Schwartz and Truman, 1983), it seemed possible that *ced-3* could encode a gene regulating the production and/or secretion of a similar hormone in worms. However, our genetic mosaic analyses of *ced-3* and *ced-4* indicated that both of these genes act cell autonomously in dying cells to control programmed cell death (Yuan and Horvitz, 1990). Thus, neither *ced-3* nor *ced-4* encodes a factor secreted to initiate programmed cell death, although it remained possible that one or both of these genes control a pathway that acts in cells that are to die in response to a secreted factor from other cells.

Because we regarded programmed cell death essentially as a cell fate, much like differentiating into a neuron or an intestinal cell, we also considered the possibility that *ced-3* acts like other cell-fate determining genes, by controlling, for example, the transcription of genes that promote a cell-autonomous death process. The discovery that *ced-3* encodes a cysteine protease indicated that it functions in a completely unanticipated way. The ability of *ced-3* to induce the death of mammalian cells in a cell-autonomous fashion (Miura et al., 1993) suggested that both CED-3 and mammalian caspases cause cells to die by a mechanism more direct than that of a hormone or a transcription factor.

The Yuan et al. (1993) paper bridged two lines of previously unrelated studies of invertebrates and vertebrates. In the late 1980s, the critical roles of IL-1β in mediating inflammatory responses made the inhibition of IL-1 signaling an intensely sought after therapeutic goal with a number of pharmaceutical and biotechnology companies attempting to purify the protease that

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processed the proform of IL-1β into the active cytokine. These efforts led to the identification of a cysteine protease, now called caspase-1, as the processing enzyme for pro-IL-1β (Cerretti et al., 1992; Thornberry et al., 1992). Structural and functional information about caspase-1 was crucial in defining its similarity with CED-3, as the sequence identity between the two is only 29%, barely significant statistically. The knowledge that the active center of caspase-1 contains the amino acid sequence QACRG, which encompasses the active-site cysteine and is also present in CED-3, made the similarity convincing. Suddenly, the efforts in the pharmaceutical industry focused on the IL-1β processing enzyme and inflammatory disease became connected to the biology of programmed cell death.

In the Yuan et al. (1993) paper, we predicted that mammalian caspase-1, or other mammalian members of the CED-3/caspase-1 family, might regulate apoptosis. This idea, which has since been confirmed by a large body of data (e.g., reviewed by Friedlander and Yuan, 1998), was surprising at the time to those studying IL-1β and its processing enzyme. There are still debates about whether or not caspase-1 and the mammalian caspases most closely related to caspase-1 are specialized for inflammatory processes or also function in apoptosis. For example, the lack of gross developmental defects in caspase-1-deficient mice (Kuida et al., 1995; Li et al., 1995) has been interpreted to indicate that caspase-1 is not directly involved in regulating apoptosis. Nevertheless, caspase-1 is activated and IL-1β levels are elevated in a variety of animal models of human diseases associated with cell deaths, including ischemic brain injury, multiple sclerosis and Huntington's Disease (Hara et al., 1997; Hisahara et al., 2001; Ona et al., 1999). Also, transgenic mice expressing an active-site mutation in caspase-1 and caspase-1-deficient mice are partially protected from neuronal cell death induced by ischemic brain injury (Friedlander et al., 1997; Schielke et al., 1998), suggesting the involvement of caspase-1 in acute neuronal cell death. Thus, caspase-1 may well be involved in regulating apoptosis under certain pathological conditions. Nonetheless, even in cases in which a deficiency in caspase-1 results in a reduction of apoptosis, such as in mouse models of ischemic brain injury, it remains possible that caspase-1 acts cell autonomously as well as by regulating a secreted cytokine(s), such as IL-1β.

In any event, there is now an established connection between inflammation and apoptosis: inflammation is generally associated with apoptosis, and apoptosis may promote inflammation (although apoptosis is not necessarily accompanied by inflammation). This connection is exemplified by the dual role of murine caspase-11, which regulates inflammation by mediating the activation of caspase-1 and apoptosis by directly activating caspase-3 (Kang et al., 2000). Such findings suggest that caspase inhibition targeting both inflammation and apoptosis may be a successful strategy for the treatment of human inflammatory diseases.

One aspect of mammalian apoptosis that could not be directly predicted by the Yuan et al. (1993) paper is the number of and interactions among caspases present in mammals. Our paper described the similarity with caspase-1 and also reported that CED-3 and caspase-1 both have similarities to the predicted protein product of the murine gene *nedd-2*, which had been identified on the basis of its high level of expression during embryonic brain development and its downregulation in the adult brain (Kumar et al., 1992). However, the published sequence of the Nedd-2 protein lacked the QACRG caspase-1 active-site sequence that was present in CED-3 and that had served as compelling evidence supporting the homology between CED-3 and caspase-1. Subsequently, the correct Nedd-2 sequence was determined and seen to include the QACRG motif, and *nedd-2* was demonstrated also to encode a caspase, named caspase-2 (Wang et al., 1994). All together, a total of 11 human caspase and 10 murine caspase family members have now been identified (Degterev et al., 2003).

While only a single caspase, CED-3, has been found to play a central role in programmed cell death in *C. elegans* (Ellis and Horvitz, 1986; Shaham and Horvitz, 1996), mammalian apoptosis pathways are executed through the concerted actions of multiple caspases arranged in protease cascades. The activation of long prodomain "initiator" caspases, such as caspase-8 and caspase-9, is often not sufficient to cause cell death, and their activities must be amplified through the activation of downstream "effector" caspases, such as caspase-3 and caspase-7 (Li et al., 1997; Srinivasula et al., 1996). Mammalian caspases, especially caspases with long prodomains, seem to be highly specialized functionally and to respond to different apoptotic signals.

Such specialization is achieved through a combination of differential protein-protein interactions and subcellular localizations. The protein-protein interactions are largely mediated via the caspase N-terminal prodomains, which contain two classes of protein-protein interaction motifs, known as DED (*d*eath *e*ffector *d*omain) and CARD (*c*aspase *a*ctivation and *r*ecruitment *d*omain) domains (Degterev et al., 2003). Caspases with DED domains, such as caspase-8, interact with the cytoplasmic domains of the Fas family of death receptors indirectly through adaptor proteins that also contain DED domains (Muzio et al., 1996) and are responsible for initiating apoptosis in response to signals from membrane receptors. The CARD domain-containing caspase-9 interacts with a specialized protein complex termed the "apoptosome," which includes Apaf-1 (a protein that is similar to the worm killer gene product CED-4) and cytochrome c and is responsible for mediating apoptotic signaling downstream of mitochondria (Zou et al., 1999).

The subcellular localization of caspases also contributes to their distinct specificities. Caspase-12 localizes to the endoplasmic reticulum (ER) and mediates ER stress-induced apoptosis (Nakagawa et al., 2000). Caspase-2 is at least partially localized to the nucleus and mediates DNA damage-induced apoptosis (Lassus et al., 2002). The functional specialization of mammalian caspases suggests that the amplification of caspases in mammalian genomes is at least in part related to the complexity of the multiple proapoptotic stimuli encountered during the life span of mammals.

A decade ago, the publication of the Yuan et al. (1993) paper helped drive the metamorphosis of apoptosis from an obscure field into a mainstream research area. The continued study of programmed cell death in *C.*

elegans has revealed that not only caspases but also many other components of the genetic pathway for programmed cell death are conserved between worms and mammals (e.g., see Horvitz, 2003). Recent discoveries about programmed cell death in *C. elegans*—for example, the finding that the process of phagocytosis, which causes cells undergoing programmed cell death to be engulfed by neighboring cells, facilitates the death process itself (Reddien et al., 2001)—remain to be explored to determine if they apply as well to mammalian apoptosis. We anticipate that future findings about *C. elegans* programmed cell death—e.g., about how specific cells decide to live or die or about the physiologically important targets of the CED-3 caspase—will continue to propel an understanding of programmed cell death in other organisms, including humans.

The impact of the Yuan et al. (1993) paper and of the studies of programmed cell death in *C. elegans* in general provide a powerful demonstration of the value of studying the biology of simple and highly tractable experimental organisms. The prophetic decision by Sydney Brenner four decades ago to select *C. elegans* as an experimental organism coupled with the landmark achievement by John Sulston of determining the complete cell lineage of *C. elegans* gave us the unique opportunity to analyze the mechanisms of programmed cell death with single-cell resolution and through the methods of analytic genetics and molecular biology. We believe that the continued study of *C. elegans* and of other simple organisms— including some that may be mostly ignored today—will continue to drive the understanding of fundamental and universal aspects of biology. We also believe that the increasing knowledge of biology that derives from such studies, including an increasing knowledge of the mechanisms responsible for apoptosis and of the many connections between apoptosis and human diseases, will lead to the development of new therapeutics that will prove of major benefit to humankind.

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