Cell, Vol. 88, 347–354, February 7, 1997, Copyright ©1997 by Cell Press

Programmed Cell Death in Animal Development

Review

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Programmed cell death (PCD) occurs during the development of all animals that have been studied, but only recently has its molecular basis been discovered. In this review, we briefly consider some of the main events in the history of PCD in animal development. We then summarize what has been learned about the molecular mechanism of PCD and some of the intracellular proteins that control it. We next discuss the functions of PCD in development and how PCD is regulated during development by signals from other cells. Finally, we consider what the evolutionary origins of PCD may have been.

Some History

Soon after it was recognized in the middle of the last century that organisms are made of cells, it was discovered that cell death can be an important part of animal development (reviewed in Clarke and Clarke, 1996). First observed during amphibian metamorphosis (Vogt, 1842), normal cell death was soon discovered to occur in many developing tissues in both invertebrates and vertebrates (reviewed in Glucksmann, 1951; Clarke and Clarke, 1996). Inhibitors of RNA and protein synthesis were later found to inhibit cell deaths that occurred during amphibian (Tata, 1966) and insect (Lockshin, 1969) metamorphosis, indicating that the deaths require macromolecular synthesis. The term programmed cell death was initially used to describe the cell deaths that occur in predictable places and at predictable times during development, to emphasize that the deaths are somehow programmed into the developmental plan of the organism (Lockshin and Williams, 1964). It was already clear, however, that some of these cell deaths can be prevented by substances released by other tissues, indicating that the deaths are not inevitable and can apparently be suppressed by signals from other cells (reviewed in Saunders, 1966).

In a seminal paper, Kerr, Wyllie, and Currie (1972) marshalled morphological evidence from studies of their own and of others to draw a clear distinction between the cell deaths that occur in both animal development and tissue homeostasis, as well as in some pathological states, and the pathological cell deaths that occur at the center of acute lesions such as trauma and ischemia. In the latter case, the cells and their organelles tend to swell and rupture in a process called cell necrosis. The leakage of the contents of the cells usually induces an inflammatory response. By contrast, when cells die during normal development or tissue homeostasis, or at the periphery of acute lesions, they usually shrink and condense, and the organelles and plasma membrane retain their integrity in a process Kerr and his colleagues named apoptosis. The dead cells or their fragments are rapidly phagocytosed by neighboring cells or macrophages before there is any leakage of the contents of the cells, and thus they do not induce an inflammatory response. Apoptotic cells in developing tissues are almost always inside other cells (Figures 1A–1C), suggesting that dying cells are usually phagocytosed before they display the morphological changes of apoptosis. Because apoptotic cell deaths usually look so similar from tissue to tissue and animal to animal (Figures 1A–1C), Kerr and his colleagues proposed that these deaths reflect the operation of an active, intracellular death program that can be activated or inhibited by a variety of physiological or pathological environmental stimuli.

It took almost another 20 years, however, before the idea that animal cells have a built-in death, or suicide, program became generally accepted, largely through genetic studies in the nematode Caenorhabditis elegans that identified genes that seem dedicated to the death program and its control (Horvitz et al., 1982; Ellis and Horvitz, 1986), and then through the finding that some of these genes were homologous to mammalian genes (Yuan et al., 1993; Hengartner and Horvitz, 1994). With this acceptance, the term programmed cell death (PCD) has come to have a different meaning from its original. It now generally refers to any cell death that is mediated by the intracellular death program, no matter what triggers it and whether or not it displays all of the characteristic features of apoptosis. It is probable that all normal cell deaths that occur in developing and mature animals, as well as many pathological cell deaths, utilize this evolutionarily conserved death program.

A cell that undergoes PCD in animal development is usually degraded so rapidly (often disappearing in an hour or less) that even when there is large-scale PCD, there are surprisingly few dead cells to be seen. This may help to explain why PCD was understudied for so long. It also suggests that the extent of PCD in animal development is still underestimated. As it is still not possible to measure clearance times in most animal tissues, quantitating PCD remains an unsolved problem.

The Death Program and Its Intracellular Control

There has been spectacular progress in the past few years in understanding the intracellular mechanism of PCD and its control, reflected in the flood of reviews celebrating this success (see, for example, Martin and Green, 1995; Chinnaiyan and Dixit, 1996; White, 1996). Rather than covering this ground again, we focus on some of the evidence that supports the following four propositions concerning the intracellular death program: (1) its protein components are constitutively expressed in all nucleated animal cells, (2) its execution seems to involve a proteolytic cascade, (3) its activation is controlled by a family of dedicated intracellular regulatory proteins, and (4) its activation during development may often be controlled transcriptionally.

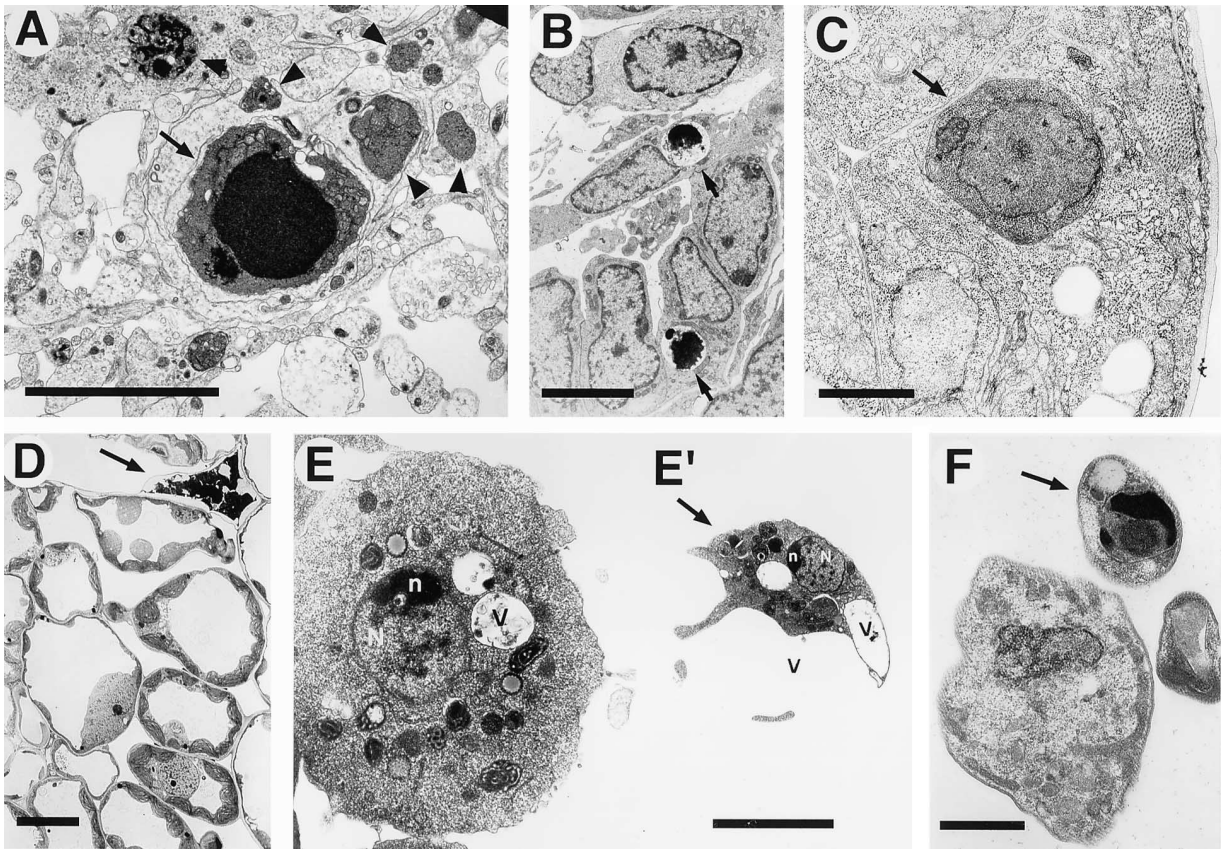

Figure 1. Electron Micrographs of Programmed Cell Deaths in Multicellular and Unicellular Organisms

Arrows point to dead cells.

(A) An early apoptotic cell in the cerebellum of a developing rat (courtesy of J. Burne). Arrowheads point to phagocytosed fragments of apoptotic cells.

(B) Two phagocytosed apoptotic cells in a developing rat kidney (Coles et al., 1993).

(C) A dead cell in the ventral nerve cord of a developing C. elegans embryo (courtesy of E. Hartwieg, G. Stanfield, and H.R. Horvitz).

(D) A dead cell within a collapsed cell wall in a tobacco leaf (courtesy of K. Plasleitt and K. Roberts).

(E and E′) A live Dictyostelium discoidium cell (E) and a dead Dictyostelium stalk cell (E′) that was induced to differentiate in culture (Cornillon et al., 1994). N, nucleus; n, nucleolus; V, vacuole.

(F) A dead and a live Trypanosoma cruzi epimastigote in culture (Ameisen et al., 1994).

Scale bars: (A), 0.5 μm; (B), 5 μm; (C), (E), and (F), 1 μm; (D), 10 μm.

One line of evidence that all nucleated animal cells constitutively express all of the proteins required to undergo PCD makes use of the drug staurosporine (STS), a bacterial product that inhibits many protein kinases (Tamaoki and Nakano, 1990). When used at micromolar concentrations, in the presence of cycloheximide to inhibit protein synthesis, STS rapidly induces PCD in all of the many nucleated mammalian cell types that have been tested. These include all of the cells that can be dissociated from a 13 day mouse embryo (after removal of its brain and liver) (Ishizaki et al., 1995) and the cells in explant cultures of a variety of neonatal and adult rodent organs (Weil et al., 1996). The only reported exception is human red blood cells (Weil et al., 1996), which do not have a nucleus or other organelles. The nucleus, however, is not required for PCD in cells that normally have one, as anucleate cytoplasts undergo PCD when treated with STS (Jacobson et al., 1994). Thus, it seems that the machinery for PCD is in place and ready to run in all of our nucleated cells, beginning with the zygote (Weil et al., 1996). Genetic experiments in both C. elegans and Drosophila suggest that the death program is also expressed constitutively in invertebrate cells (Steller, 1995; Shaham and Horvitz, 1996), making it likely that this is a basic feature of all nucleated animal cells.

The most important clue to the molecular nature of the death program came initially from genetic studies in C. elegans that identified a gene called *ced-3* that is required for the 131 PCDs (see Figure 1C) that occur during the development of the worm (reviewed in Ellis et al., 1991). The gene encodes a cysteine protease that is homologous to interleukin-1β-converting enzyme (ICE) (Yuan et al., 1993), a mammalian cysteine protease that produces the proinflammatory cytokine IL-1β from its precursor protein. At least 11 members of the Ced-3/ICE family of proteases have now been identified in humans, and a number of them have been implicated in PCD (reviewed in Chinnaiyan and Dixit, 1996; Kuida et al., 1996). All cleave their substrates after specific aspartic acids and are themselves activated by cleavage

at specific aspartic acids. They are now therefore referred to as caspases (for cysteine aspases, see Alnemri et al., 1996). At least in vitro, some caspases can activate themselves, and some can activate other caspases, suggesting that they probably act in a proteolytic cascade (see Nagata [1997, this issue of *Cell*]). Caspases mediate PCD by cleaving selected intracellular proteins, including proteins of the nucleus, nuclear lamina, cytoskeleton, endoplasmic reticulum, and cytosol. Some of the cleaved proteins activate other destructive processes in the cell and thereby help kill the cell neatly and quickly (Chinnaiyan and Dixit, 1996). As specific protein or peptide caspase inhibitors can block PCD in all animal cells that have been tested, it seems likely that caspases form the core of the death program in all animal cells, although some of them, such as ICE, have other functions.

At least one of the intracellular mechanisms that controls the death program in animal cells has also been conserved in evolution. The *ced-9* gene, which acts to inhibit PCD in C. elegans (Hengartner and Horvitz, 1994), is homologous to the *bcl-2* gene, which acts to inhibit PCD in mammalian cells (Vaux et al., 1988; reviewed in Korsmeyer, 1995). The human *bcl-2* gene is even able to inhibit PCD in the worm (Vaux et al., 1992; Hengartner and Horvitz, 1994). A number of Ced-9/Bcl-2 family members have been identified in mammals. Some, such as Bcl-2 and Bcl-X_L, inhibit PCD, whereas others, such as Bax and Bak, promote PCD. The various family members can dimerize with one another, with one monomer antagonizing or enhancing the function of the other. In this way, the ratio of inhibitors to activators in a cell may determine the propensity of the cell to undergo PCD (Korsmeyer, 1995), although the activity of some of these proteins can also be regulated by phosphorylation (reviewed in Gajewski and Thompson, 1996). It is still not known how any of these proteins operate. The three-dimensional structure of Bcl-X_L suggests that it may function as a pore-forming protein in the intracellular membranes where it is found (Muchmore et al., 1996). Whatever their mechanism of action turns out to be, it is clear that they play a crucial role in regulating PCD in development. If *ced-9* is inactivated by mutation, for example, most cells in the developing worm undergo PCD, and the worm dies early in development (Hengartner et al., 1992). It seems that Ced-9 is required to keep the death program off if a cell is to survive in the developing worm. Similarly, if either *bcl-x* (Motoyama et al., 1995) or *bcl-2* (Veis et al., 1993) is disrupted in mice, the animals die as embryos or postnatally, respectively, as the result of excessive PCD in particular organs. Conversely, if *bax* is disrupted, some normal PCDs fail to occur (Deckwerth et al., 1996).

Although all of the proteins required to execute the death program seem to be constitutively expressed in animal cells, inhibitors of RNA or protein synthesis often inhibit PCD, indicating that transcription and translation are often required to activate the program. The strongest evidence that new gene transcription is involved in activating PCD during normal development comes from experiments in Drosophila, where linked genes (*reaper*, *hid*, and *grim*) required for triggering PCD are transcriptionally activated 1–2 hours before the cells die. If these genes are inactivated, none of the normal cell deaths occur and the flies die early in development (White et al., 1994). It is not known how the products of these genes activate the caspase-dependent death program in developing fly cells. Although homologous genes have not yet been found in other organisms, it seems likely that many of the PCDs that occur during the development of other animals are also transcriptionally controlled (Tata, 1966; Lockshin, 1969; Martin et al., 1988; Oppenheim et al., 1990).

Functions of PCD in Animal Development

How important is PCD in animal development? Mutant nematodes that are PCD-deficient can have a normal lifespan in the laboratory, even though they have about 15% more cells than normal and function less well than wild-type worms (Ellis et al., 1991). PCD-deficient flies, by contrast, die early in development (White et al., 1994). At least in terms of complexity, vertebrates are more similar to flies than to worms, and it seems likely that they would die early in development if their cells could not undergo PCD. Consistent with this view, mice in which CPP32 (caspase-3) has been deleted by targeted gene disruption die perinatally with a vast excess of cells in their central nervous system, apparently as a result of decreased PCD in neuroepithelial cells, although PCD in other organs seems to occur normally (Kuida et al., 1996). PCD serves many functions in animal development, and these have been classified in different ways (see, for example, Glucksmann, 1951; Horvitz et al., 1982). We consider five functions, most of which involve eliminating unwanted cells: (1) sculpting structures; (2) deleting unneeded structures; (3) controlling cell numbers; (4) eliminating abnormal, misplaced, nonfunctional, or harmful cells; and (5) producing differentiated cells without organelles.

PCD plays an essential role in sculpting parts of the body. The formation of digits in some higher vertebrates is a well-studied example, where PCD eliminates the cells between developing digits (Figure 2A). If the cell death is inhibited by treatment with a peptide caspase inhibitor, digit formation is blocked (Milligan et al., 1995; Jacobson et al., 1996). Similarly, PCD is involved in hollowing out solid structures to create lumina (Figure 2B). In early mouse embryos, for example, the preamniotic cavity is formed by the death of the ectodermal cells in the core of the developing embryo (Coucouvanis and Martin, 1995). PCD also occurs wherever epithelial sheets invaginate and pinch off to form tubes or vesicles, as in the formation of the vertebrate neural tube or lens, and it is observed when two epithelial sheets come together and fuse, as in the formation of the mammalian palate (Glucksmann, 1951). It is not known, however, if cell death is required for any of these processes, although it seems likely that it plays an important part.

In the course of animal development, various structures are formed that are later removed by PCD. These include vestigial structures that were required in an ancestral species but not in the descendant, structures that are needed at one stage of development but not later (Figure 2C), and structures that are required in one sex but not in the other. Pronephric tubules, for example,

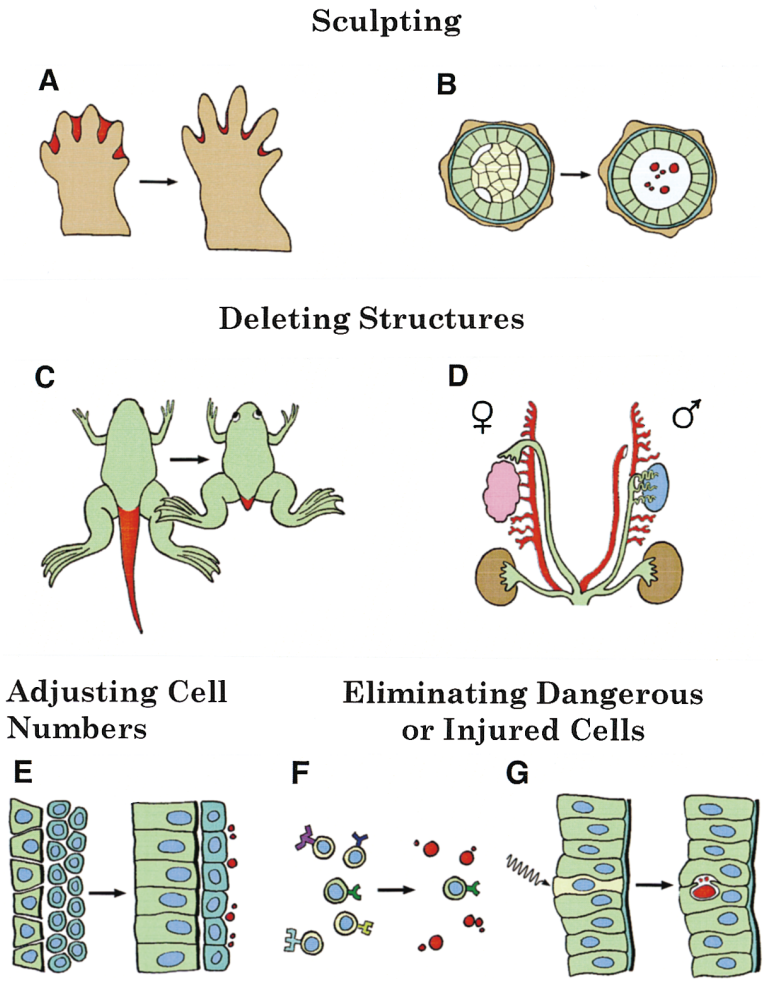

Figure 2. Some Functions of PCD in Animal Development

(A and B) Sculpting.
(C and D) Deleting unwanted structures.
(E) Controlling cell numbers.
(F and G) Eliminating nonfunctional, harmful, abnormal, or misplaced cells.

form functioning kidneys in fish and amphibian larvae, but they are not used in mammals and are eliminated by PCD. Subplate neurons are required transiently during the development of the mammalian cerebral cortex and are subsequently removed by PCD. The Müllerian duct forms the uterus and oviducts in female mammals, but it is not needed in males and is thought to be lost by PCD. Conversely, the Wolffian duct forms the vas deferens, epididymis, and seminal vesicle in males, but it is not needed in females and is eliminated by PCD (Figure 2D).

In many organs, cells are overproduced and then culled by PCD to adjust their numbers (Figure 2E). In the vertebrate nervous system, for example, both neurons and oligodendrocytes are generated in excess, and up to half or more are eliminated by PCD, apparently to match their numbers to the number of target cells they innervate (Barde, 1989; Oppenheim, 1991) or the number of axons they myelinate (Barres et al., 1992), respectively. Although the influence of cell proliferation in controlling cell numbers in animal development has received more attention than the influence of PCD, PCD can be the dominant mechanism. In well-fed hydra, for example, cell proliferation greatly outstrips cell death, and new hydra continually bud off from the parent animal; when hydra are starved, growth stops, mainly because cell death greatly increases, while the rate of cell proliferation changes very little (Bosch and David, 1984).

PCD also functions as part of a quality-control process in animal development, eliminating cells that are abnormal, misplaced, nonfunctional, or potentially dangerous to the organism. Striking examples are seen in the vertebrate immune system, where developing T and B lymphocytes that either fail to produce potentially useful antigen-specific receptors or produce self-reactive receptors that make the cells potentially dangerous are eliminated by PCD (Figure 2F). Animal cells have poorly understood ways of recognizing when they are damaged and will undergo PCD if the damage is great enough (Figure 2G). If DNA is damaged sufficiently in a mammalian cell, for example, the cell can activate its death program by various mechanisms, one of which depends on the p53 tumor-suppressor protein (Clarke et al., 1993; Lowe et al., 1993). This response not only serves as an anticancer mechanism, but also seems to help prevent the birth of defective offspring. If pregnant mice are irradiated, the number of offspring produced decreases, as many of the irradiated embryos die, but there is surprisingly little increase in the occurrence of birth defects among those mice that are born. Mouse embryos lacking both copies of the p53 gene, however, tend not to die, and many are instead born with abnormalities (Norimura et al., 1996).

The death program may be involved in producing specialized differentiated cells without organelles. Certain cell types, including skin keratinocytes, lens epithelial

cells, and mammalian red blood cells, lose their nucleus and other organelles in the process of terminal differentiation. The differentiated lens cells and red blood cells continue to live in the sense that they continue to metabolize, whereas differentiated keratinocytes die and form a layer of corpses (squames) on the surface of the skin. There are hints that these highly specialized differentiation processes may be modified forms of PCD. The nuclei become pyknotic, the DNA becomes fragmented, and, in the case of human keratinocytes in culture, overexpression of a *bcl-2* transgene inhibits terminal differentiation (Nataraj et al., 1994). It remains to be determined, however, whether caspases are involved in any of these differentiation processes.

There are many cell deaths that occur during animal development where the function is unknown. This is the case for the cell deaths that occur in the inner cell mass of early mammalian embryos (El Shershaby and Hinchliffe, 1974), in developing mammalian spermatogonia (Allan et al., 1987), and among undifferentiated proliferating cells in many developing organs. Why should these cells die before they have had a chance to function? It is possible that some of these deaths reflect a continuing competition between developing cells for a limited supply of extracellular survival signals, which may serve to constantly adjust cell numbers and may, at the same time, select for the "best" cells (Raff, 1992, and see below). The use of caspase inhibitors may make it possible to determine the functions of some of these enigmatic cell deaths.

Extracellular Controls on PCD in Development

Why do cells die when and where they do during development? The deaths could, in principle, reflect cell-autonomous controls, but because the death program can be activated or suppressed by extracellular signals from other cells, they could also reflect the operation of death-activating signals or the failure to receive adequate death-suppressing signals. In most cases where the control mechanism is known, signaling between cells is involved. We first consider examples where developmental cell deaths result from insufficient death-suppressing signals and then discuss examples where they are induced by death-activating signals.

There is increasing evidence that most developing cells, at least in higher animals, require signals from other cells to avoid PCD (reviewed in Raff, 1992). Much of the evidence comes from in vitro experiments, where it is found that most vertebrate cells undergo PCD when cultured on their own at low density unless serum or exogenous signaling molecules are added to the culture medium. A well-known exception is blastomeres, which can survive and divide in the absence of signals from other cells (Biggers et al., 1971). Although most cells require signals from other types of cells to survive in culture, some produce survival signals for themselves: this is the case for cells from tissues that are composed of only a single cell type, such as lens (Ishizaki et al., 1993) and cartilage (Ishizaki et al., 1994). For most cells, a combination of several types of signaling molecules is required for long-term cell survival in culture. These can be soluble, bound to the plasma membrane, or bound to the extracellular matrix.

Although few studies have examined the survival requirements of cells in vivo, those that have suggest that cells in developing vertebrate tissues also require signals from other cells to avoid PCD. Furthermore, they suggest that many normal cell deaths during development reflect the failure of the cells to receive such signals. Many types of developing vertebrate neurons, for example, are thought to compete for limiting amounts of survival signals (neurotrophic factors) secreted by the target cells they innervate. Only about half of the neurons get enough signal to survive, while the others undergo PCD (reviewed in Barde, 1989; Oppenheim, 1991). Developing sympathetic neurons, for instance, depend on nerve growth factor (NGF) that is released by their target cells. If developing animals are given exogenous NGF, the normal death of sympathetic neurons is prevented (reviewed in Levi-Montalcini, 1987). Conversely, if they are treated with anti-NGF antibodies (Levi-Montalcini, 1987), or if the genes that encode NGF or its receptor (trkA) are inactivated (reviewed in Snider, 1994), all of these neurons undergo PCD. This dependence on limiting amounts of target cell–derived survival signals is believed to be a mechanism for matching the number of neurons to the number of target cells they innervate, as well as for eliminating neurons that connect to inappropriate target cells. It seems likely that similar mechanisms operate in nonneural tissues to ensure that cells only survive when and where they are needed (Raff, 1992).

The dependence of animal cells on survival signals from other cells probably explains many of the ectopic cell deaths that occur when normal development is perturbed. As both the availability of survival signals and the survival requirements of a cell change as the cell develops, cells that fail to develop normally will probably not receive the signals they need to survive. Moreover, cells that develop normally but depend for their survival on cells that do not may also die.

Some cell deaths that occur in animal development are triggered by PCD-inducing signals, which can act systemically or locally and override the action of survival signals. A classic example of systemic induction is seen in amphibian tadpoles at metamorphosis, where an increase in thyroid hormone in the blood induces cells in the tail to undergo PCD, facilitating the resorption of the tail (Kerr et al., 1974). Examples of local induction have been described in developing chick embryos, where NGF induces PCD in cells of the early central retina (Frade et al., 1996) and bone morphogenic proteins (BMPs) induce PCD both in prospective neural crest cells before they migrate from rhombdomeres 3 and 5 (Graham et al., 1994) and in interdigital cells that are eliminated during digit formation (Gañan et al., 1996; Zou and Niswander, 1996).

For the great majority of cell deaths that occur in normal development, however, it is not known whether or how extracellular signals are involved in the deaths. In many cases, a combination of both PCD-activating and PCD-suppressing factors probably determines which cells survive and which die. In the early mouse embryo, for example, PCD-activating signals from the visceral endoderm apparently induce embryonic ectoderm cells to die, thereby forming the proamniotic cavity. Those embryonic ectoderm cells that are sitting

on the basal lamina, however, seem to be protected from these lethal signals by survival signals associated with the basal lamina (Coucouvanis and Martin, 1995). Having the death of a cell depend on both the presence of a PCD-activating signal and the absence of a survival signal allows for greater control than if one of these mechanisms operated alone.

Evolutionary Origins of PCD

Where did the caspase-dependent death program in animal cells originate? Did it arise with the first animals, the first multicellular organisms, or some unicellular organism? Although neither caspases nor Ced-9/Bcl-2 homologues have been found in nonanimal cells, many nonanimal cells seem to have death programs of some kind.

Cell death plays a crucial part in plant development, for example, in the formation of xylem, flowers, and ovules; it also occurs in the senescence of flowers and leaves and in the hypersensitive response of plants to pathogens (Greenberg, 1996). In the green algae Volvox, the simplest of plants, the synchronous death of all of the somatic cells early in development allows the juvenile gonidia to hatch out and prepare for a new round of reproduction. Although the cell deaths in plants can share some morphological features with PCDs in animals (Levine et al., 1996), the presence of a cell wall means that the dead cells cannot be phagocytosed in a plant (see Figure 1D). The molecular mechanism(s) of plant cell death is unknown.

Cellular slime molds are thought to have descended from an ancestral eukaryote that ultimately gave rise to plants, fungi, and animals (Loomis and Smith, 1995). They display both unicellular and multicellular forms in their life cycle. Dictyostelium discoideum, for example, proliferates as free-living amoeboid cells when conditions are favorable. When starved, the cells aggregate and develop into a fungus-like structure, consisting of a stalk and a fruiting body, which contains large numbers of viable spores. The stalk cells are dead, having died as a normal part of their differentiation program, and they display some features in common with apoptotic animal cells (Cornillon et al., 1994) (Figure 1E), but the mechanism of the cell death is unknown.

Even some eukaryotic organisms that remain unicellular throughout their life cycles may have a death program. Trypanosomes, for example, are unicellular parasites that can cause serious disease in animals, including humans. They have recently been shown to die with many of the characteristic features of apoptosis when they fail to differentiate into trypomastigotes in culture (Ameisen et al., 1995) (Figure 1F). Although yeasts do not display a death program during their normal life cycle, when the PCD-promoting Ced-9/Bcl-2 family member Bax is expressed in budding yeast, it kills the cells, and the coexpression of Bcl-2 blocks this death (Hanada et al., 1995). The morphology of the dead cells does not resemble apoptosis, however, and the significance of this finding is unclear.

Surprisingly, the most convincing and best-characterized cell death programs in unicellular organisms are seen in prokaryotes, where they seem to have arisen as a consequence of a competition between bacteria and their viruses, between one strain of virus and another, or between bacteria themselves. The molecular mechanisms are remarkably varied. Some work by cleaving RNA or DNA, others by cleaving specific proteins, and still others by inserting pores into the plasma membrane (reviewed in Snyder, 1995). There is one suicide program in some strains of E. coli that works much like PCD in animal cells in that it is based on a constitutively expressed proteolytic enzyme, which is activated in response to infection by a T4 phage. When activated by the binding of a protein in the head of the phage, the enzyme cleaves the translational elongation factor EF-Tu, arresting translation and thereby killing the cell (Yu and Snyder, 1994). By committing suicide in response to infection, the bacterium may protect its nearest neighbors, which are likely to include its genetically identical siblings. The protease is encoded by a defective prophage, suggesting that this suicide program may have initially arisen in an evolutionary battle between bacterial viruses (Shub, 1994). It is possible that a mechanism such as this in an ancestral prokaryote may have provided the starting point from which the death program in animal cells eventually evolved.

Acknowledgments

We thank P. Golstein, R. Horvitz, A. Mudge, and R. Parnaik for helpful comments on the manuscript; J. Scholes for providing the drawings for Figure 2; and J.-C. Ameisen, J. Burne, P. Golstein, R. Horvitz, K. Plasleitt, K. Roberts, and G. Stanfield for providing electron micrographs. Because of the limited number of references allowed, we were unable to cite many important papers; we apologize to their authors.

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