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Review

Programmed cell death 50 (and beyond)

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In the 50 years since we described cell death as 'programmed,' we have come far, thanks to the efforts of many brilliant researchers, and we now understand the mechanics, the biochemistry, and the genetics of many of the ways in which cells can die. This knowledge gives us the resources to alter the fates of many cells. However, not all cells respond similarly to the same stimulus, in either sensitivity to the stimulus or timing of the response. Cells prevented from dying through one pathway may survive, survive in a crippled state, or die following a different pathway. To fully capitalize on our knowledge of cell death, we need to understand much more about how cells are targeted to die and what aspects of the history, metabolism, or resources available to individual cells determine how each cell reaches and crosses the threshold at which it commits to death.
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Facts

- 50 Years ago we suggested that cell death was 'programmed' or written into the developmental pattern of cells.
- Since that time we have come to understand the phenomenon of apoptosis, to understand its relationship to many diseases including cancers, neurodegenerative diseases, and disorders of the immune system, and to understand its biochemistry, genetics, and immediate means of control.
- We have also recognized alternative patterns in which cells die, some of which (autophagy) in normal circumstances serve to protect cells against death.
- Nevertheless, cells vary in response to inducers or blockers of cell death.
- Most of what we study are end-stages of longer processes that involve the metabolism and history of the cells, as well as their interactions with other cells and environment. We need to know more about these stages.
- With the availability of new, high-resolution techniques, we should be able to explore these aspects as well.

Open Questions

- Each cell has a distinct history and metabolism, so that each cell responds differently in timing and response to the same stimulus. We need to know much more about what determines the threshold at which the cell death mechanism is activated.
- Autophagy appears to be a response to penury and often, initially, protects cells against toxic stimuli. When, in developmental situations, this process starts in otherwise seemingly healthy cells, we need to know what triggers it.
- To what extent are alternative forms of cell death part of a continuum and to what extent are they unique?
- What functions do caspases have in healthy cells, and how are they controlled?
- At what points in the cell death process will it be appropriate to intervene for therapeutic purposes?

It is rather humbling to have the opportunity to write this commentary. In many ways, one has the sense of being a fossil, and in this case a very particular type of fossil: the remnants of an oyster shell at 2700 m at the top of the Grand Canyon (see, for instance, Cutler, A, The Seashell on the Mountaintop, 2003, Dutton. There are remnants of marine organisms near the top of Mt. Everest: http://www.npr.org/2012/03/16/148753432/mount-everest-still-holds-mysteries-for-scientists or http://en.wikipedia.org/wiki/Mount_Everest). Geologists and evolutionists remark it, and even tourists come to see it, but what is really interesting, and most remarkable, is what happened after the oyster settled into the mud – how the seashore where it lived rose to 2700 m above sea level. The point of seeing the oyster is to marvel at the process that lifted it. As is the case with the oyster, for the field of cell death the people, the processes, the discoveries, and the insights that lifted the field so high are the true story. Looking at the oyster, the tourist or scientist should stare at the rock and marvel at the process – that he or she is not, in fact, feeling the spray of the ocean. But, to continue the metaphor, the story of the building of the research field of programmed cell death or apoptosis quickly becomes too vast, too global, and too powerful to easily contemplate and contain. That story is contained in other reviews.[1–7]

Also, as in geology and the bit of former shoreline that one observes, the beginning of the research field is an arbitrary point, not necessarily fixed with the publication of the first papers entitled 'programmed cell death'. 'Programmed cell death' was inherently a metaphor, a felicitous turn of phrase designed to exploit the trendiness of the then-nascent

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computer era. The intent was to focus attention on what was relatively obvious: that cell deaths in developing and metamorphosing animals occurred at predictable developmental stages and in specific locations. They must be 'programmed' into the genetics of the organisms, in the same sense that the differentiation and growth of an organ, tissue, structure, or pigment would be considered to be fundamentally determined by the interplay of specific genes. In the 1960s, we could breed *Drosophila* to select some genes but had no such ability with insects large enough to be subjected to surgical or other experiments, and no real ability to manipulate specific genes in any eukaryotic organism. I, under the direction of Carroll M. Williams and at his suggestion, set about to find components of the control system that determined the loss of larval tissues and organs at metamorphosis. We chose to follow the fate of the intersegmental muscles of American silkmoths. These large and powerful abdominal muscles survive from the larva into the pupal phase, and serve to force hemolymph (the insect's circulatory fluid) into the wings of the freshly emerged adult, thus expanding them. Silkmoths do not feed as adults and the muscles, which can account for 2–3% of the fresh weight of the insect, are destroyed and recycled within 48 h of escape from the cocoon. Because the muscles disappear in the adult, we could store pupae for experimentation throughout the year, and our experiments were relatively less complicated by massive simultaneous changes such as those occurring during pupation. In our first papers, we identified endocrine, neural, and neurosecretory components of the program; the neurosecretory components, adumbrated at the time, were far more thoroughly documented by James Truman[8–10] a few years later. Within the muscles, we also observed a massive expansion of the lysosomal system before the time at which the muscles show signs of deterioration and ultimately depolarize, which we interpreted to indicate a preparation for and progress toward death.[11–15] Cholinergic toxins, if administered before or immediately after the neurosecretory signal, could block the progress toward death. Later, we demonstrated a requirement for new protein synthesis.[16] Although this latter was an exciting discovery, ultimately it became obvious that synthesis was required only for developmental situations such as for the intersegmental muscles, involuting tadpole tails,[17] and differentiating central neurons.[18]

However, back to the metaphor: Others chose to recognize the description of 'programmed cell death' as a starting point, the site where the oyster existed and defined the shoreline or beginning on the field. If we looked further, we could have identified a shoreline that had appeared in the late nineteenth century, when several histologists and anatomists noted in passing that cells died in many developmental and reproductive situations;[2,3] in the 1940s, when Viktor Hamburger and Rita Levi-Montalcini[19–23] recognized a substantial difference in size of sympathetic and sensory ganglia between regions of the body innervating limbs and those that did not. They determined that the difference in size resulted from the death of immature neurons in the trunk regions, and the survival of neurons in regions containing limbs. We could have also considered the 1950s to be a starting point, when Dame Honor Fell[24,25] noted matter-of-factly that chondrocytes in culture differentiated themselves into death; and when John Saunders started to examine patches of cells that died to sculpt the limbs of chick embryos ('necrotic zones')[26,27] and the literature on cell death had collected enough observations, although scattered and unfocused, that the radiologist Glücksmann chronicled a list of 74 multiply reported instances in which cell death had been documented.[28] Glücksmann categorized these deaths according to their purported utility or purpose in the life of the organism. We would today consider that type of characterization to be antiquated, but the basis of his argument was that cell death was clearly a normal and distinctly not pathological aspect of the life cycle.

What has happened since that early period has been amply described in many reviews[1–7] (see Figure 1) and need not be further elaborated here. However, we can look at where we stand and where we are likely to go. For a summary of the history of interest in the field, see Figures 2 and 3.

A rather important concept, and one that is often ignored, is that cell death is a process, but it is the end phase of a larger process (Figures 4 and 5). That is to say, healthy cells do not spontaneously die and, in spite of the ease of the verbal shorthand, they do not 'decide to die'. Cells are not sentient beings. They have strong negative biochemical and molecular feedback loops to maintain stability within defined physiological limits, and they have specific positive feedback ('feedforward') processes that guarantee that, should those limits be breached or threatened, the cell will destroy itself in a controlled manner with minimal damage to the organism. We know best the means of activation of these feedforward mechanisms, such as the interaction of a TNF-family ligand with its receptor ('extrinsic activation of apoptosis') or the release of cytochrome *c* and apoptosis initiating factor from the mitochondria ('intrinsic activation of apoptosis'). The extrinsic activation of apoptosis is easily comprehended within the context of the organism: it is a physiological (organism-level) control whereby one cell or type of cell issues a death sentence for another, to combat a serious situation such as an embryonic hematopoietic stem cell that produces anti-self antibodies,[29,30] a viral infection, or a cell that has escaped the normal social controls that maintain homeostasis,[31] or simply to bring an excess burden of cells, such as lymphocytes at the end of an infection, to a level more in equilibrium with the host requirements. Sometimes, disastrously, mistakes are made. In terms of human health, these mistakes can produce developmental anomalies, cancers, autoimmune disorders, and neurodegenerative disorders, as well as, potentially, more subtle and slower-developing disorders arising from an imbalance in homeostasis.[32] We therefore are developing a medical armamentarium to address these mistakes.

The situation for the control of intrinsic apoptosis is more complex. It is typically initiated by an impending metabolic failure and triggered by mitochondrial destabilization before too much energy has been drained to prevent apoptosis – and therein lies the rub. First, it is highly likely that caspases have non-death-related functions in healthy cells[33] and therefore may theoretically be active without killing the cell. Depending on the kinetics, at one extreme the metabolic problem that threatens the cell may resolve itself and the cell will survive, whereas at the other extreme the cell may lose control of its ionic pumps before it has completed apoptosis and, accumulating lactic acid, undergo osmotic lysis (necrosis).

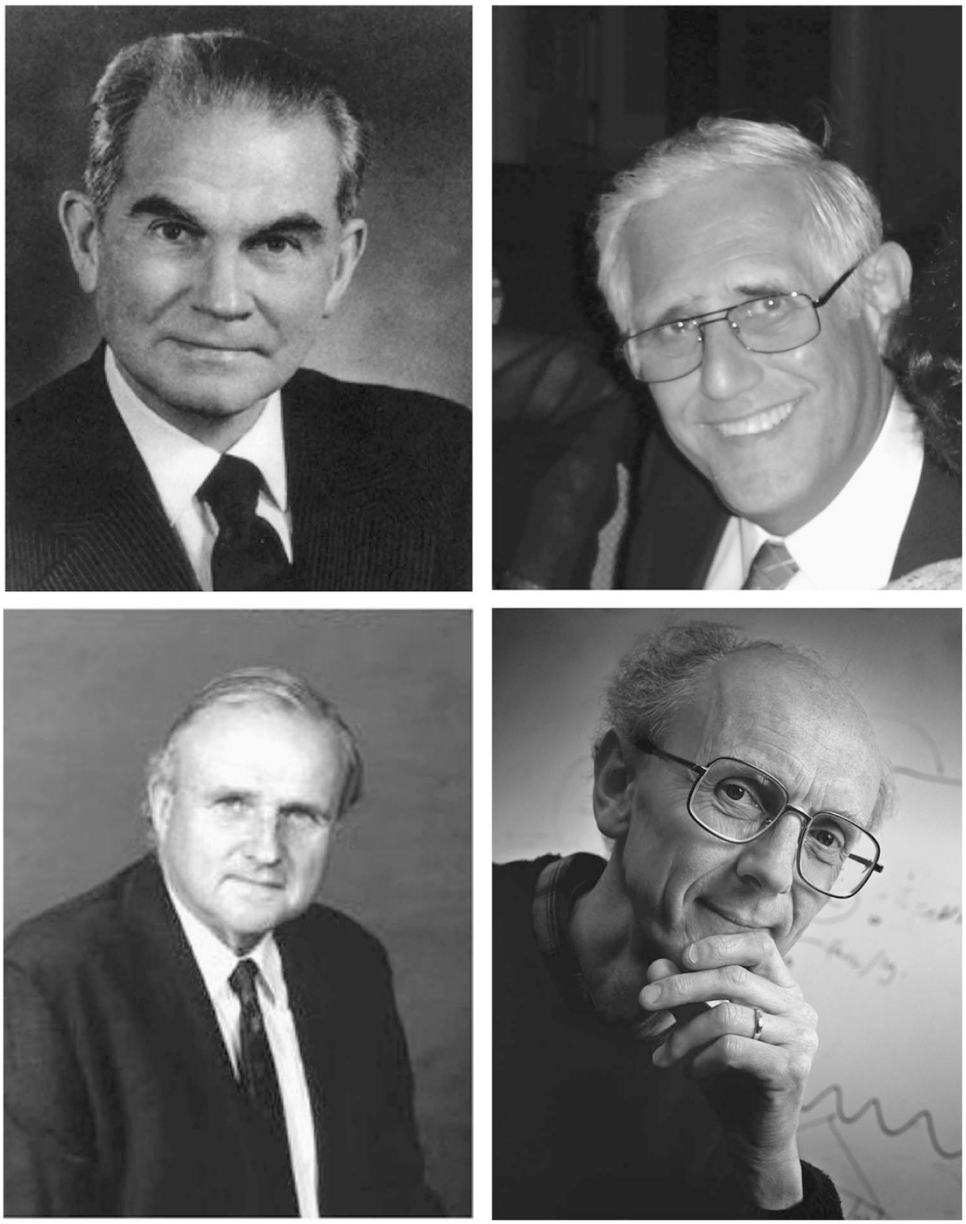

Figure 1 Originators of 'Programmed Cell Death' and 'apoptosis'. Top row, left: Carroll M Williams, circa 1970 (courtesy of Lynn M Riddiford); Richard A Lockshin, circa 2010 (Wikipedia); John FR Kerr, circa 2000, courtesy John Kerr); Andrew Wyllie, circa 1995, copyright James King-Holmes, all rights reserved. For more contemporary photos, including other authors important to the field, see the study by Lockshin and Zakeri[4]

In between are alternative possibilities, including necroptosis,[34] pyroptosis,[34] ferroptosis,[35,36] and others. Although each of these is interesting and perhaps addressable for itself, one must not overlook the essential point: the affected cell is in agony, and its metabolic feedback loops are adjusting to address the imbalance as evolution has selected them to do. Unless we are looking only to temporarily control an acute situation, such as limiting cell death in cells at the penumbra of the immediately affected area following a heart attack or stroke, or restricting the immediate impact of an otherwise highly toxic chemotherapeutic drug, then we must ultimately address the stresses on the impacted cell.

One situation that most clearly illustrates the question of whether a cell responds to a physiological insult or provocation by dying or surviving is the interaction between autophagy and apoptosis. In the late 1950s, de Duve and his collaborators discovered lysosomes.[37,38] The discovery was accidental: they had developed the technique of differential centrifugation and, having left the fractions of a liver homogenate overnight rather than processing them immediately, they found much more acid phosphatase in the 'mitochondrial' fraction. They quickly determined that what we now know as primary lysosomes had ruptured, releasing acid phosphatase and other acid hydrolases into the supernatant. Recognizing that

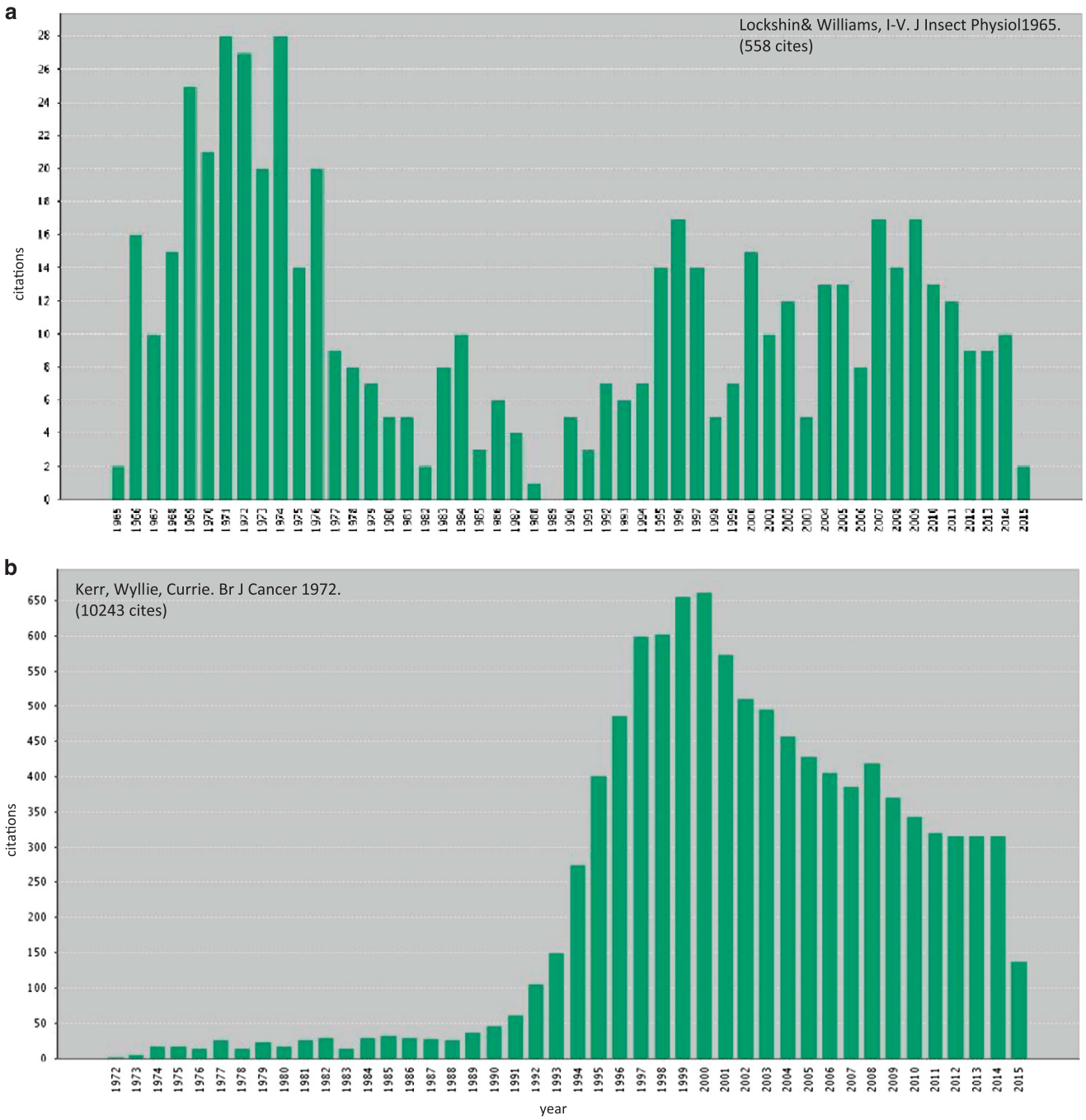

Figure 2 Citations of Lockshin and Williams (I-V)[11–15] and Kerr *et al.*[44] The early interest in Lockshin and Williams (upper graph) reflected citations primarily in the literature of developmental biology, pathology, pharmacology, and insect physiology. It resurged in the 1990s as interest in apoptosis grew and three of the original five articles were eventually incorporated into the PubMed indices. Likewise, interest in 'apoptosis' (lower graph) was modest until advances near 1990 (see the text) stimulated interest in the field. Supplied by an unnamed reviewer

the acid hydrolases were potentially dangerous to the cell, they hypothesized that the rupture of lysosomes could kill a cell and tested the hypothesis by intoxicating the liver of a rat with a known and commonly used hepatotoxin, carbon tetrachloride (CCl_4). As they quickly determined, CCl_4 caused the membranes of the lysosomes to rupture, and lysosomes were given a name reflecting their putative function. As we now know, CCl_4 is a lipid solvent and dissolves or damages all cell and intracellular membranes.

Nevertheless, lysosomes were an exciting topic of research, and no alternatives were being considered as mechanisms of cell death. Although Kerr questioned how a dying cell could shrink and condense;[39] we and others recognized the validity of the question;[40] and Cidlowski *et al.* attempted to analyze the mechanism,[41,42] most of the focus of cell death research was on lysosomes and the lysosome family, including autophagosomes and autophagic vacuoles. Most of these studies involved large, sedentary, post-mitotic, or minimally mitotic

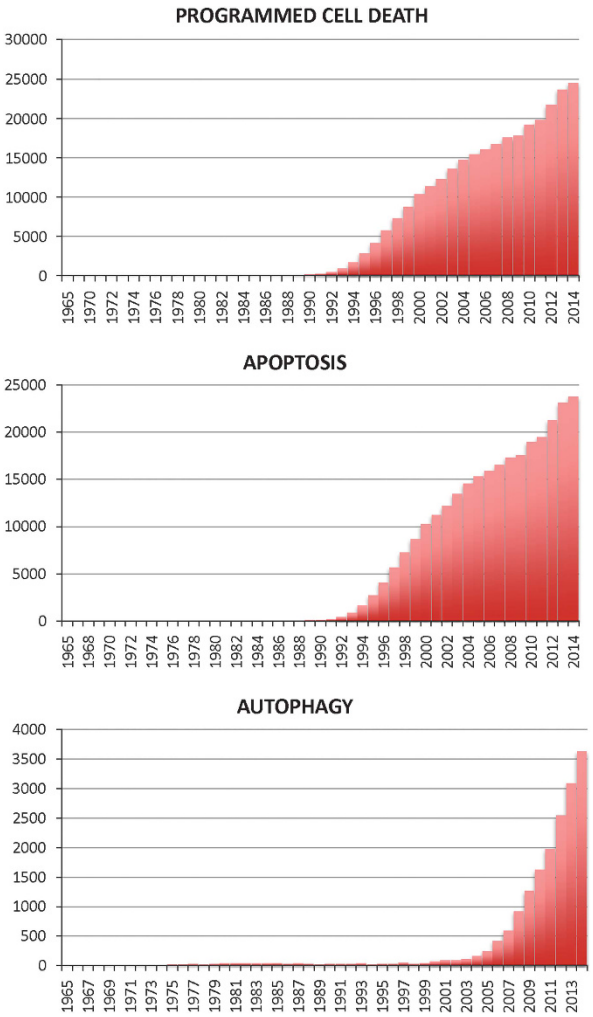

Figure 3 Citations of 'Programmed Cell Death,' 'Apoptosis,' and 'Autophagy'. There was modest interest in the first two topics until the early 1990s, and the terms were considered synonymous from some point in the first decade of the twenty-first century, resulting in considerable overlap. Although 'Autophagy' evoked some interest from the 1970s, interest did not begin to rise until approximately 2000, when the genetics of autophagy was elucidated and its distinction from apoptosis was emphasized

cells such as muscles, mammary epithelium, and prostatic epithelium[4,43] as opposed to cells with large nuclei and more modest amounts of cytoplasm, derived from highly mitotic progenitors such as thymocytes, lymphocytes, and their progenitors and relatives. Later, after Kerr, Wyllie, and Currie had generalized the concept of apoptosis, primarily using these cells;[44] Wyllie and his collaborators had demonstrated a cheap and technically easy means of assessing apoptosis;[45] the genetics of apoptosis were defined (see Horvitz[46] for summary); and several cancers were recognized to be driven by mutations in machinery controlling apoptosis,[4] large numbers of researchers and clinicians queried the nature of apoptosis. Thus, in the early 1990s, apoptosis became a topic of intense interest, surpassing autophagy as the primary focus for cell death researchers.

The hypothesis that cells died by autophagy ('autophagic cell death') was already problematic. It was known that autophagy was a normal part of the metabolism of healthy cells, accounting for the turnover of organelles and other cell constituents, and there was no obvious dividing line between this routine function and one in which autophagy could kill a cell. In most circumstances, autophagy was self-limiting but in others it appeared to account for the demise of the cell. For instance, PC12 cells can be differentiated into neurons in the proper conditions, including the presence of NGF. Once they acquire neuronal morphology, they become dependent on NGF, and will die if it is removed. In this case, autophagy continues until mitochondria are destroyed, thus depriving the cells of any possibility of recovery.[47–49]

The genetic analysis of autophagy in yeast, primarily by Klionsky and others,[50,51] the extension of the genetics to mammalian cells, primarily by Levine,[52–54] and the development of new tools to study lysosomes, including fluorescent markers[55] and lysosome-specific drugs[56,57] have clarified the situation considerably and allowed much closer examination of what autophagy does. It now appears that autophagy usually protects cells[5,58] in that cells that can activate autophagy withstand many types of stresses far better than cells that cannot.[59,60] Cells and viruses struggle for the control of autophagy, each for their own teleonomic purposes.[59] Often the virus stimulates autophagy in the infected cell, generating resources and staving off apoptosis until the virus reproduces. The protein components of autophagic and apoptotic pathways can interact: autophagy can destroy damaged mitochondria or proteins signaling endoplasmic reticulum stress before they can activate apoptosis,[61] and caspases can destroy proteins that would otherwise activate autophagy.[60,62] Thus. today's consensus is that autophagy is a response to stress or damage.[63–67] Activation of autophagy serves to eliminate the damaged material and to generate extra energy, allowing a cell to survive a hopefully transient stress. If, in spite of this protection, the cell is too greatly stressed, it will undergo apoptosis. If apoptosis is for some reason blocked (through mutation, inhibitor, or virus[62]), the cell can continue autophagy until it finally destroys itself. This consensus is a hypothesis, as was the hypothesis that autophagy was activated to kill cells. It can change again.

All this takes place within a cell that presumptively neither plans its future nor considers its relationship to the organism. In the mechanistic view of cell biology, biochemical and biophysical changes within the cytoplasm beget adjustments that activate autophagy, apoptosis, or other responses. So, taking as a model an involuting tissue or gland such as an insect labial or salivary gland at metamorphosis or post-lactational mammary epithelium, all instances of this 'runaway autophagy,' we reach some fundamental questions: first, what are the stresses on the cell, and what are their origins? What limitations do changes in hormones, growth factors, or physical properties impose on the cell? In insects, the cells of metamorphosing organs and tissues are exposed to ecdysone in the absence of juvenile hormone, and perhaps changes in delivered nutrients. The changes in nutrients, ions, cytokines, paracrine materials, and other material in circulation may result from the contemporary metamorphosis of other tissues. Mammary epithelium experiences a sharp drop in prolactin as well as engorgement of cells from synthesized, unreleased milk. Neurons depend on support from glial cells

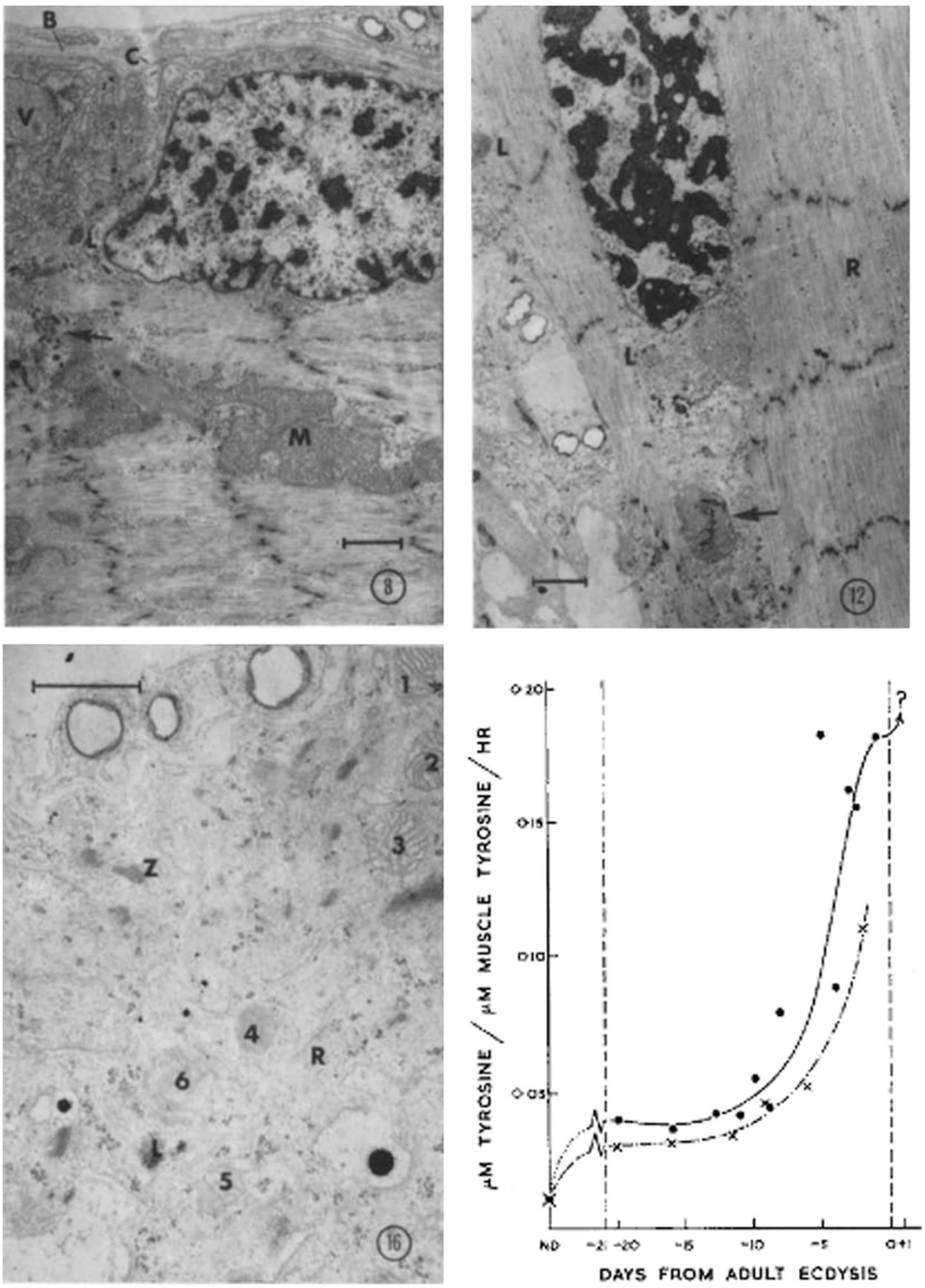

Figure 4 Some of the original evidence for programmed cell death. Upper left: Electron micrograph from a moth intersegmental muscle immediately after eclosion (hatching) of the moth, at which time the muscle is fully intact and functional. This is the normal appearance of an insect nucleus. B: basement membrane; C: plasma membrane; L: lysosome-like object; M: mitochondrion; V: synaptic vesicles. Upper right, equivalent view, 10 h after eclosion, showing beginning erosion of myofilaments, lysosomes, and beginning condensation of chromatin, which occurs sporadically and is not well-developed until much later. n: nucleolus; R: sacroplasmic reticulum; arrow: degenerating mitochondrion. Some myofilaments are disoriented. Lower left: Similar muscle 15 h after eclosion, showing substantial erosion of myofilaments, many lysosomes, and shrunken but otherwise intact mitochondria. R: remnant of sarcoplasmic reticulum; Z: Z-line; 1–6; mitochondria in various stages of deterioration. In all micrographs, scale line = 1 μm. Lower right: Increase in a lysosomal enzyme, cathepsin D, in intersegmental muscles of two species of silkmoth from the beginning of adult development (day 0 at left) to eclosion (day 0 at right). This early increase was one of the arguments for programming. Electron micrographs from the study by Lockshin and Williams;[14] graph from the study by Lockshin and Williams[12]

and on a continuous supply of NGF; they are in trouble if either is limited. Are these stresses unique and original, or are they related to other stresses, but in this instance stronger or unrelieved for too long a time?

These questions are dramatic for these specific situations, and they remain important for all studies of cell death. Even under our most carefully controlled experiments, not all cells die, or do they die simultaneously. Something about individual

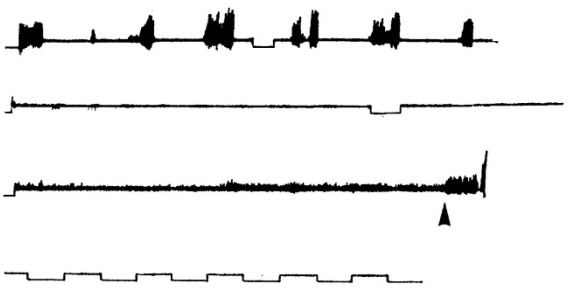

TABLE 2—EFFECTS OF PILOCARPINE ON THE DEGENERATION OF INTERSEGMENTAL MUSCLES

Stage at injection*	Number of surviving preparations	Percentage of moths showing: Complete retention	Partial retention	No retention
Pharate adult	75	85	13	1
0–6 hr after ecdysis	41	37	27	37
6–12 hr after ecdysis	10	0	0	100

Figure 5 Other evidence for physiological control of death of insect intersegmental muscles. Upper: recorded spontaneous activity from nerves innervating the intersegmental muscles shortly before and 3 1/2 h after eclosion. The spontaneous activity decreases sharply within 2 h. From Lockshin and Williams:[15] Lower: Effect of cholinergic drugs (here pilocarpine) administered to insects within the first hours after eclosion. Within the first 5 h after eclosion, pharmacological stimulation of the central nervous system could prevent the muscles from degenerating as measured by dissection after 4 days. From Lockshin and Williams[13] (Endocrine control of the degeneration was addressed in the study by Lockshin and Williams[11])

cells – their current metabolic reserves, their antecedent history, or distance from mitosis, the proximity of other cells that can support or undermine them, or many other factors – makes it possible for cells to respond differently to the same stress.[5,68–74] When we attempt to change their fate, it is not sufficient to consider that we can block or induce apoptosis. Cells have far more options than apoptosis, especially when we consider their behavior in an organism rather than in a Petri dish. If the stress remains, the cell is still likely to die or survive in an atrophied state. If it has a function such as secretion or maintenance of a high resting potential, that function may be lost.[75–79]

Ultimately, we need to know through what pathways each stress percolates through the cell, and how these pathways interact with each other. In intact mammals, the ability to mount an immune response is relevant [80,81] This is a complex task, and the more we confront it, the better discrimination we will have between pathological and healthy tissues. Understanding why a mitochondrion fails, or what determines where and when an autophagic vacuole forms, is our most immediate new goal.

With each generation come new tools and new approaches. We have at least one billion-fold greater sensitivity than 50 years ago, enabling us to conduct experiments of which we could not even dream at the time. With techniques such as fluorescence and nano-technologies, ultra-resolution microscopy, high-throughput gene screening, the ability to transiently up- and downregulate genes, and PCR-based quantification of transcriptional activity, we can learn the minutest details of cell behavior. To understand it all, however, we need always to view each biological process in the context of what else is happening within the cell, what options the cell has, and the context in which the cell finds itself within the organism. Because of all the new possibilities and new discoveries, the future in this field looks to be even more exciting than the last 50 years. It remains a joy to feel that one is a scientist.

Conflict of Interest

The author declares no conflict of interest.

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