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PROGRAMMED CELL DEATH AND APOPTOSIS — WHERE IT CAME FROM AND WHERE IT IS GOING: FROM ELIE METCHNIKOFF TO THE CONTROL OF CASPASES

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The story of cell death began with the origins of cell biology, including important observations by Elie (Ilya) Metchnikoff, who realized that phagocytes engulfed dying cells. Most of the early studies were observational. By the middle of the 20th C, researchers were beginning to explore how cells died, had recognized that cell death was a physiologically controlled process, that the most common mode of death ("shrinkage necrosis", later apoptosis) was tightly controlled, and were speculating whether lysosomes were "suicide bags". Just prior to 1990 several discoveries led to rapid expansion of interest in the field and elucidation of the mechanisms of apoptosis. Closer to the beginning of the 21st C comprehensive analysis of the molecules that controlled and effected apoptosis led to the conclusion that autophagic processes were linked to apoptosis and could serve to limit or increase cell death. Today, realizing that knowledge of the components of cell death has not yet produced pharmaceuticals of therapeutic value, research is turning to questions of what metabolic or other mechanisms indirectly control the activation or suppression of the cell death positive feedback loop. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".

***Key Words*: programmed cell death, apoptosis, caspase, history, autophagy, lysosomes.**

In one sense, the story of cell death has some relationship to the Ukraine. Cell death plays a major role in development, homeostasis, and pathology but, although dying cells were recognized almost as soon as techniques permitted the examination of cells, its importance was largely underestimated because dying cells were so rarely seen. They are rarely seen because, while the newly synthesized DNA of a dividing cell can be labeled and that division can be identified as long as the cell survives, perhaps for many years, a dying cell is identifiable for 20 minutes to one hour, after which it is gone. It is gone because, most typically, it has been consumed by a phagocytic cell or phagocyte. Phagocytes were of course first described and documented — against considerable disbelief — by one of The Ukraine's gifts to world science, Ilya (Elie) Metchnikoff. In fact, through the history of the field, the story of cell death pops up periodically as a factor in our understanding of cell biology.

In the mid-19 century, the German dye chemists were discovering that many plant, animal, and mineral extracts colored various fabrics. As can be seen in many paintings, the variety of colors of dresses and garments increased remarkably during that period. Scientists realized that these same dyes could color the frustratingly transparent cells that they were trying to study. Thus the science of histology was born, and, almost as soon as scientists could see cells, they realized that some of the cells would die. In 1842, Karl Vogt, studying the metamorphosis of amphibians, realized that the notochord disappeared, and he indicated that the death of the notochord cells had to be physiological [1]. In 1864, August Weissman, also in metamorphosis, pointed out the death of most cells in the pupae of insects [2]. He introduced the term "histolysis", and described the appearance of dying cells. During the remainder of the 19th century, many other cells were observed to die during normal development or metamorphosis: chondrocytes, cells in the ovarian follicle, post lactational mammary glands, myocytes and myofibrils, sensory neurons, and many others. Even Metchnikoff, looking at the number of phagocytes in the regressing muscles of the tadpole tail, pointed out that the muscles were in fact dying [3]. Throughout the first third of the 20th century, there were many studies of cell death, mostly in embryos and metamorphosing animals. The first mention of cell death appears in the mid-19th C, with the writings of the great Walther Flemming [4], who described the involution of Graafian follicles in mammals, followed by incidental reports by others on what appeared to be dying cells in the nervous system and elsewhere. In the beginning of the 20th C, a noticeable interest in metamorphosis of insects and amphibians, primarily in France, led to several publications, marvelous for their length and for their elaborate line and water-color drawings, particularly of the destruction of musculature and nervous system in insects and tadpoles at metamorphosis. Biologists like von Recklinghausen [5] had even clearly distinguished between oncosis, what we would today term necrosis, and the more physiological cell death that most commonly is described as apoptosis. However, techniques were very limiting. Most cell deaths were observed in small tissues or animals, over a very short period of time, and only a small percentage of the cells could be dissected free. Thus the scientists at that time did not dare to hope that

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they would be able to understand the causes or mechanisms of the deaths that they observed.

Starting in the 1930s, the situation began to change. Victor Hamburger was exploring the mechanisms whereby tissues stimulated the growth of neurons [6] — the story that later would become the story of nerve growth factor. A few years later, he, together with Rita Levi-Montalcini, would clearly demonstrate that many neurons were born in each sensory ganglion, but in the absence of supporting tissue generating nerve growth factor, many of the neurons that were born would soon die [7]. Honor Fell, later Dame Honor Fell, began to examine in cell culture how chondrocytes died [8]. And John Saunders, recognizing specific patches of dying cells in chick embryos, wondered if he could dissect them free and study the control of their death [9]. Likewise, immunologists began to recognize that many thymocytes died in mammalian embryos, and even as the white blood cell count fell after an infection, this drop in white blood cells derived from the death of the circulating cells. In 1951, Alfred Glücksmann published a major review listing nearly 100 types of cell death during early vertebrate development [10]. Though his classifications are more focused on the purpose of the deaths, which today we would find not very helpful, he very effectively demonstrated that all cell death was a very normal aspect of development and homeostasis.

The 1950s bore witness to a major expansion in the technology of cell biology. Microscopy was rapidly improving with the development and rapid growth of the capabilities of the phase contrast and electron microscopes, and the technology of homogenizing, osmotically protecting, and separating cell organelles by differential centrifugation was rapidly coming on board. Christian de Duve and his collaborators were examining the properties of mitochondria, which they could remove and purify by differential centrifugation. One enzyme that they considered to be mitochondrial was acid phosphatase. One night, they inadvertently left their samples on the desktop, rather than returning them to the refrigerator for storage overnight. They nevertheless tried to use their samples the next day and found, to their surprise, that the acid phosphatase activity was hugely increased. This led to their discovery of lysosomes and their exploration of the property of these organelles [11]. They learned that the lysosomes were distinct from the mitochondria and that they contained many acid hydrolases. Trying to discover the function of the lysosomes, they exposed rats to carbon tetrachloride, a known hepatotoxin, and looked at the effect on the lysosomes of the liver. They therefore proposed that the lysosomes were suicide bags, killing cells when they ruptured. We know today that this result was specific to carbon tetrachloride, since this lipid-soluble toxin directly attacks cell and lysosomal membranes. Nevertheless, it was the first hypothesis concerning the mechanism of cell death. As we describe below, this was one lead that we followed.

Meanwhile, John Saunders was becoming curious about the mechanisms by which cells died. He took cells from the axillae of embryonic chicken wings, which would die in the near future, and explanted them into tissue culture. They did well in the tissue culture until the time that they would have died in the embryo, at which time in culture they died. One could say that the cells were already moribund at the time he explanted them, but Saunders demonstrated that this was not true, for, when he transplanted them not to a culture dish but to the back of another embryo, they healed in and survived, contributing to the epidermis on the back of the host. Thus, as he would observe later, the cells were not already dying, but "the death clock was ticking" [9].

Personal comments RAL ["When I entered graduate school, my potential mentor, Carroll M. Williams, suggested a series of possible projects to me. One was the fate of larval tissues during metamorphosis. Because he could store pupae in the refrigerator and take them out year around, he suggested that the death of the intersegmental muscles in the freshly emerged adult would be a non-season-limited tissue on which I could work. Since my undergraduate degree was in biochemical sciences, I considered that I could test the hypothesis of suicide bags. However, it is never a good idea for a graduate student to bet on only one horse — what if it doesn't work? — and I elected to consider also neurological and endocrine mechanisms, and to invest some time in looking at the tissues by electron microscopy. Briefly, what we learned was the following:

- For the muscles to die, they needed to be potentiated by the initial endocrine signal that led to the metamorphosis of the adults. If one interfered with that, one could also interfere with the death of the muscles [12].
- As the development advanced and the insect approached ecdysis, the number of lysosomes in the tissue began to increase rapidly [13].
- At the moment of emergence of the adult from its cocoon, there was a neural signal later (demonstrated by Truman et al. [14] to be also neurosecretory) that triggered the conversion to the active phase of death. Chemically or surgically removing the neural activity led to the premature death of the muscles, while chemically or electrically driving the activity prevented the death of the muscles at the appointed time [15, 16].
- Shortly after the activity of ecdysis ceased, there was a rapid expansion of the lysosomal system, including the development of autophagic vacuoles and autophagosomes, and death became irreversible.
- As we were to learn later, the events immediately surrounding ecdysis required synthesis of new messenger RNA and protein and could be blocked by administration of drugs that inhibited these processes [17].
- Also determined later, during the first 8 to 12 hours after ecdysis, the ensuing death was occult; the muscles were physiologically normal and could contract and respond normally. After 12 hours, by which time approximately ⅓ of the myofilaments had already been resorbed, the muscles rapidly

depolarized, became non-contractile, and were quickly resorbed [18].

Carroll Williams was always known for his colorful phraseology, and as graduate students we always tried to emulate him. Because computers were just beginning to be talked about at the time, programmed cell death seemed to be a particularly modern and colorful way of describing what we saw. It was a metaphor stating what I thought was pretty obvious — if a biological process occurs at a defined location and time, then it must in some fashion be programmed or written into the genetics of the organism — but, as in poetry, metaphors help people see things that they otherwise would have not have noticed. Thus a relatively straightforward observation gained some currency"].

Meanwhile, with growing improvement in technical ability, many other laboratories beginning to investigate the mechanisms of cell death. To a very large extent, these studies focused on very appearance, number, and changes in morphology, of lysosomal bodies. In 1996 Jamshed Tata, exploiting the recent discovery that actinomycin D could inhibit the synthesis of proteins, established that protein synthesis was required for the death of explants of tadpole tail [19]. Lockshin confirmed the findings for insect muscle [17], as did Munck and White [20] for find thymocytes exposed to glucocorticoids. Likewise, Oppenheim confirmed that protein synthesis was necessary for the death of neurons in chick embryos [21]. (This requirement later proved to be restricted to embryonic and other immature cells, as opposed to post mitotic or differentiated cells, but the findings provoked more interest in the mechanisms of cell death.) Another paper that provoked some interest was that of Kerr, Wyllie, and Currie [22]. In this paper the authors drew on Kerr's earlier observations that "shrinkage necrosis" was common to most physiological forms of cell death [23] and that, unlike osmotic lysis in necrosis, there was no good mechanistic explanation of how it occurred, and they proposed that this type of cell death was the complement of mitosis, and suggested the name "apoptosis". We were becoming more comfortable with the idea that death was not a random event, but rather a controlled event, for which mitosis was the compensation.

However, what really launched the field was a group of three scientific advances. Fittingly, each was of a different type: technical, conceptual, and theoretical.

The technical breakthrough was that in 1990, Arends and Wyllie, expanding Wyllie's earlier interest in chromatin rearrangement in apoptosis, published a paper indicating that the simple technique of electrophoresing DNA could demonstrate apoptosis, since in necrotic cells randomly degraded DNA would produce a smear, whereas in apoptosis the DNA was cut in a more orderly fashion, between nucleosomes [24]. The technique and its expansion to more sensitive (labeling) versions was simple and cheap, enabling many laboratories to look for apoptosis. Spontaneous apoptosis hard to spot — the entire liver is replaced in approximately 3 years, and an apoptotic cell may be identifiable for only 20 minutes, thereafter leaving no trace, whereas with appropriate labels a mitotic event may be traced years later. Even at the turnover rate of the liver, only one cell in 72.000 would be apoptotic at any time. Thus a technique that could reveal apoptosis allowed many researchers to observe apoptosis in many pathological and non-pathological (e.g., expansion and contraction of immunocompetent cells) situations, and to convince the research community of its importance.

The conceptual breakthrough was the recognition that several diseases, notably cancers, were associated with abnormal patterns of cell death. Thus in rapid succession B-cell lymphoma was recognized to be a failure of lymphocytes to die on time, deriving from the translocation of a gene that prevented cell death (Bcl-2) to a position of constitutive activation [25, 26]. The gene p53, known to be mutated in the majority of cancers, had been assumed to act by preventing mitosis of cells in which DNA had been damaged, but was now recognized to provoke apoptosis if these cells had left G_0 [27, 28]. Finally, a cell surface protein, variously named Fas Ligand, Apo-1, and CD95, was recognized as capable of killing cells when linked to a soluble or cell-bound component [29, 30]. Peter Krammer displayed spectacular pictures of tumor regression when the ligand was engaged. Ameisen [31] suggested that the devastation of AIDS was generated by the death of not seriously infected bystander cells, which might be prevented. Thus cell death in general, and apoptosis in particular, were recognized as being important in medicine.

The theoretical breakthrough was the realization that the mechanism of cell death, and even the components of cell death, were conserved in evolution from nematode worms to mammals. Thanks primarily to research coming from the laboratory of Horvitz and collaborators, the primary effector gene of apoptosis in worms, ced-3, was identified as a protease very similar to mammalian proteases, leading to the discovery of a new class of proteases (caspases) mostly associated with apoptosis [32]. The basic mechanism of control — all mature cells containing an inactive protease capable of killing the cell; the protease is activated by an activating molecule that interacts with it; and the activator is held in check by other molecules that normally suppress it — is common to worms and mammals. Even the components are evolutionarily related: ced-3 to caspases, Bcl-2 to ced-9, and Apaf-1 to EGL-1. This conservation argues for a highly important biological role for cell death. The history is summarized in the Table. The basic pathways of apoptosis, summarized from many sources, are shown in Fig. 1, and the formal and molecular relationships of worm and mammalian apoptosis are illustrated in Fig. 2.

In the 21st century there have been many advances, too numerous to describe, and which have been reviewed in many other competent reviews. In brief, these can be summed into two categories: first, now that apoptosis is well understood, it has become possible and to recognize many other forms of cell death. Second, although we have considerable understanding

of the mechanisms of cell death, medical interventions based on this understanding has not been forthcoming.

The first category illustrates a common problem in the history of science: Although autophagy was considered a major mechanism of cell death in the 1970s, and was extensively studied, excitement over apoptosis led to the presumption that apoptosis was the only meaningful form of cell death. By the beginning of the 21st century, it was apparent that not all forms of cell death represented classical apoptosis. There were of course the situations in which cells differentiated into nonviable forms, such as lens epithelium, keratinocytes, platelets, and erythrocytes. Several laboratories recognized that sometimes cells begin apoptosis but fail to complete it, since apoptosis require energy and it is possible to exhaust that energy before apoptosis is completed. Some forms of cell death were clearly programmed but more closely resembled necrosis and were given names such necroptosis and paraptosis. To some extent, the biology determined the situation: the normal fate of an apoptotic cell is to be phagocytosed, but if phagocytes cannot reach the apoptotic cell, it may end in a form of necrosis. More problematic is the situation in insect metamorphosis. Although insect cells like those other animals can undergo apoptosis, in metamorphosis apoptosis is not seen. Instead, the cells undergo massive autophagy before fragmenting and being consumed by phagocytes. This situation obtains also for large mammalian cells with substantial cytoplasm, such as mammary epithelium in post lactational stage. Such situations gave rise to the concept of autophagic cell death.

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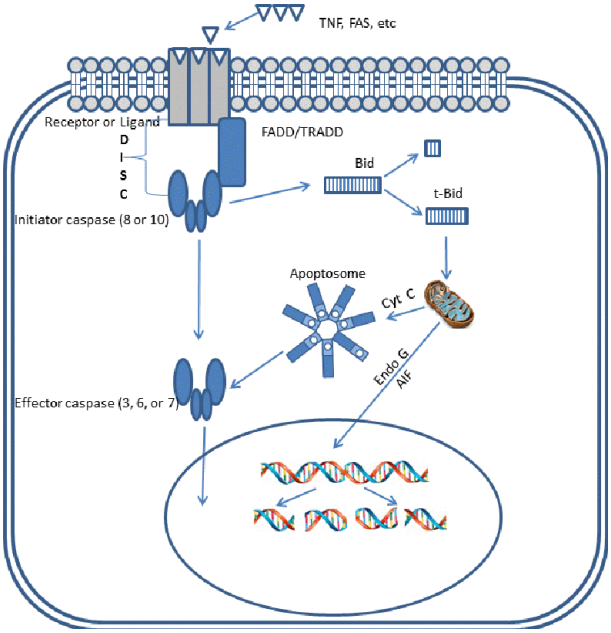

Fig. 1. Primary routes of apoptosis. Apoptosis may be initiated commonly by two routes: In the first (top) or extrinsic route an extracellular molecule (Tumor Necrosis Factor — TNF) or Fas interacts with a membrane receptor, activating the Death Initiating Signaling Complex or DISC, which includes a defined sequence called the Death Domain (Fas-associated Death Domain FADD/TNF-Receptor with a Death Domain TRADD). In the complex an initiator caspase (cysteine protease hydrolyzing at aspartic acid), caspase 8 or 10, is activated. This in turn activates an effector caspase, caspase 3, 6, or 7, which destroys many cytoplasmic proteins and, by entering the nucleus, destroys chromatin and nucleoplasmic proteins, permitting cleavage of DNA. In the second, or intrinsic, route, the initiator caspase may cleave Bid (BH3 interacting domain death antagonist) to produce truncated Bid (t-Bid), which as a pro-death molecule, can compete with death antagonists at the mitochondrial outer membrane. Alternatively, metabolic conditions may also lead to the same mitochondrial outer membrane. In any event, the mitochondrion leaks cytochrome C, endonuclease G, and apoptosis-initiating factor (AIF) to the cytoplasm. In the cytoplasm, cytochrome C and AIF bind to and activate a multimeric complex termed the apoptosome, thus activating another initiator caspase, caspase 9. Caspase 9 then activates the same effector caspases

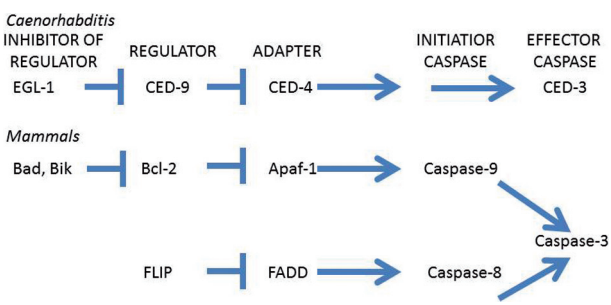

Fig. 2. Parallelism of *Caenorhabditis* and mammalian pathways of apoptosis. In these widely divergent creatures death is effected by a caspase. In mammals the effector caspase is activated by initiator caspases. The caspase is activated through interaction with an adaptor, but the adaptor is normally held in check by a regulator. In certain cells at certain times, the normally-on regulator is blocked by a pro-death protein that releases the death pathway. The worm proteins Ced-9, Ced-4, and Ced-3 have not only formal but partial structural similarity to the corresponding mammalian genes

Today the situation for autophagy seems far more complex. Heavily stressed cells, such as those infected by with viruses, use autophagy as a defense mechanism. Those stressed cells that can undergo autophagy survive better than those that cannot. If we were to reassess the situation today, it would appear that autophagy is normally a defense mechanism that under some circumstances continues until all necessary resources of the cell are consumed and the cell finally dies. What controls the onset and limitation of autophagy, and why the cell does not initiate apoptosis, are unknown and worthy of further study.

Finally, the reason that cell-death-based therapy is not yet available is becoming clear. For the most part, the origin of cell death based disease is not a failure of the mechanism of cell death — that is, of the caspases or other controllers of death. The problem is normally the inappropriate activation or failure of activation of the cell death mechanism. Other than the case of B cell lymphoma, in which the Bcl-2 gene is translocated to a position in which it is constitutively active, the problem is not with the effectors themselves but rather the threshold at which cell death is activated. We do not yet understand what determines the threshold or sensitivity of the cell. Identifying and targeting these thresholds will ultimately produce cell death controlled therapy.

This essay has tried where possible to cite some of the originators of our current thinking in their original publications. Several histories have been published that give expanded or alternate views and are well worth reading in their own right. These reviews include those by Clarke and Clarke [62], Lockshin and Zakeri [63], Vaux [64], and Lizarbe Iracheta [65]. Some of the material in the tables and figures reflects observations originally made by these authors.

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