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Minireview

Biochemical events in naturally occurring forms of cell death

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Several molecular elements of programmed cell death and apoptosis have recently been revealed. The function of gene products which deliver the lethal 'hit' is still not known. Well-characterized and newly discovered cell surface structures (e.g. antigen receptors, *FAS/APO-1*), as well as transcriptional factors (steroid receptor, c-*myc*, *P53*, retinoblastoma protein and others), have been implicated in the initiation of the death pathway. Negative regulators of the process (*ced-9* gene product in programmed death of cells in *Chaenorhabditis elegans* and *bcl-2* protein in apoptosis) have been described. Biochemical mechanisms responsible for the silent nature of natural deaths of cells include their rapid engulfment (mainly through integrin receptors), transglutaminase-catalyzed cross-linking of cellular proteins, and fragmentation of DNA. Several lines of evidence suggest that distinct molecular mechanisms may operate in various forms of natural cell death.

Lethal switch; Death control; Negative regulator; Silent removal; Distinct form

1. INTRODUCTION

The intense genetic, biochemical and cellular studies of recent years have revealed the existence of a basic, evolutionarily conserved mechanism of regulated cell death which usually occurs under physiological conditions and is often programmed [1–3]. It is generally agreed that a cascade of genes must be expressed in order for the cells to die. During evolution, the death program has been modified in a cell- and tissue-specific manner to accomplish a number of physiological functions. These include elimination of cells that (i) have no function, (ii) generated in excess, (iii) develop improperly, (iv) have already completed their lifespan or (v) are harmful [1], and (vi) production of dead cells for specific function (e.g. cornification and lens cells [3]). Genetic analysis in *Caenorhabditis elegans*, in which one of eight nuclei generated in the normal lineage of the hermaphrodite is fated to die (always the same cells at the same time of development), has identified several genes that function in normal death [1]. The biochemical function of the products of these genes is not known. However, a number of biochemical events have been observed during apoptosis [3,4], a natural death phenomenon originally described in mammalian cells by morphological criteria [2,5]. The combined evaluation of recently acquired molecular data points to critical elements of programmed cell death, as well as apoptosis, and suggests that diverse biochemical pathways may lead to the ultimate fate of cells.

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2. THE LETHAL SWITCH

The existence of killer gene products has been clearly shown in *C. elegans* where the loss of *ced-3* and *ced-4* activity allows cells, which normally die, to live [1]. The normal products of these genes, which act within the cells themselves, must activate programmed cell death or participate directly in the killing. According to DNA sequences they are novel proteins; the *ced-4*-encoded one contains two sites similar to the Ca^{2+}-binding EF-hand motif [6], and the *ced-3* protein contains many serine residues in motifs similar to known phosphorylation sites [1]. Neither the regulation nor the biochemical activity of these proteins has been determined yet.

Chromatin fragmentation into oligonucleosomal length fragments (which appear as a 'ladder' after DNA electrophoresis) by a non-lysosomal Ca^{2+}/Mg^{2+}-dependent endonuclease (which is constitutively expressed in almost all types of cells) has long been considered to be the hallmark, and potentially even the initiating mechanism, of apoptosis [7]. However, in *C. elegans*, genes for cell death are clearly separable from, and act prior to, an endonuclease (the product of the *nuc-1* gene) which acts in cells engulfing and degrading the dead cells [1]. In three other instances of programmed cell death the activation of an endonuclease has been shown to be neither a trigger nor a necessary or defining component of the early phase of death [8]. Similarly,

condensation of the chromatin at the membrane of apoptotic nuclei, an early morphological sign of apoptosis, have not been found associated with activation of an endonuclease [9,10], and several types of cells undergo apoptosis without DNA fragmentation [11]. It has recently been demonstrated that the Ca^{2+}/Mg^{2+}-dependent endonuclease is functionally and antigenically identical to DNase I [12]. It has been suggested that, in addition to increased intracellular Ca^{2+} levels, lamin phosphorylation and solubilization, and breakdown of the nuclear envelope, are early events in cell death, allowing the rapid access of activated endoplasmic reticulum enzymes, such as the DNase I, to the nucleus. The well-known actin–DNase I interaction might be of physiological relevance during mitosis and apoptosis; whereas the free DNase I is kept inactive during the nuclear membrane breakdown in normal cell cycle by G-actin, this control may be impaired during cell death. It should be noted that there are reports suggesting that apoptosis is actually linked with DNA structural degradation of one strand [13] and that selective cleavage of 28 S rRNA variable regions is one of the critical elements of apoptosis [14].

The elusive nature of the killer gene products makes studying their regulation and control difficult. In most of such studies DNA fragmentation, which now seems to be neither a specific nor a critical event of the process, has usually served as the quantitative and/or qualitative measure of apoptosis or even programmed cell death. Nevertheless, a number of initiating mechanisms are now known. Binding of ligands, such as transforming growth factor β_1, antigen and tumor necrosis factor (TNF), to their respective cell surface receptors initiates apoptosis in appropriate cell types (for references see [3]). *FAS/APO-1* is a transmembrane member of the TNF receptor family that mediates apoptosis [15,16]; its expression on target cells is necessary for them to be killed by cytotoxic T cells [17], and its mutation leads to autoimmune disease [16]. A significantly conserved novel protein domain at the cytoplasmic region of the *FAS/APO-1* antigen and type I TNF receptor seems to transduce the apoptotic signal into susceptible cells [18]. The signal pathways (which may include Ca^{2+}, cAMP, protein kinase C, phosphatases and kinases other than protein kinase C [3,4,19,20]) and the molecular details of the transcriptional response of cells to receptor stimulation, vary from one cell/receptor type to the other. It is determined by the maturation and differentiation status of the cell, i.e. how much they are competent and primed for apoptosis [3,4].

Glucocorticoids, retinoic acid and thyroxin induce apoptosis in susceptible cells through the transactivating potential of their respective nuclear receptors [2,3]. The c-*myc* proto-oncogene, usually implicated in cell transformation, differentiation and cell cycle progression, also has a central role in some forms of apoptosis. The c-*myc* gene product, when over-expressed in cells deprived of growth factors [21] or treated with heat [22], induces apoptosis and inhibition of c-*myc* expression with anti-sense oligonucleotide prevents activation-induced death of T cell hybridomas [23]. There is evidence that the T cell activation-induced transcriptional factor *Ap-1* (homo- or heterodimer of members of the Jun and Fos family) is involved in programmed cell death [24,25]. The continuous expression of c-*fos* at sites of naturally occurring cell death of *fos–lacZ* transgenic mice, beginning hours or days before the morphological demise of the cell, appears to be a hallmark of terminal differentiation and a harbinger of death [26]. Over-expression of the wild-type *p53* anti-oncogene product could induce apoptosis in cultured cells [27], and thymocytes from strains of *p53* 'knock-out' mice are extraordinarily resistant to the induction of the process by radiation and topoisomerase II inhibitors [28,29]; intranuclear *p53* accumulates following DNA damage, and this is part of the lethal signal. Mice deficient in the retinoblastoma oncosuppressor gene show accentuation of the normal tissue patterns of apoptosis, particularly in the central nervous system, which leads to the death of these animals in utero (Andrew Wyllie, personal communication). The well-known activity of all of these proteins in transcriptional regulation points to their involvement in turning on, and modulation of, one or more 'lethal' genes; the identity of such genes and their relationship to cell death events is completely unknown.

3. CONTROLLING DEATH

There is increasing evidence to suggest that most animal cells are capable of killing themselves [1,30,31]. Since the program of self-elimination is built into the biochemical machinery of cells it should be tightly controlled. Cells require survival signals to stay alive. These signals are provided by survival factors acting on cell surface or nuclear receptors (growth factors, antigens, hormones, cytokines, bacteria; see [3,31]) and by differentiation. Survival of most mammalian cells is temporary since only a few types live throughout the lifetime of the organism. What is the biochemical explanation of cell survival?

Since the regulation of the putative death switch seems to be mainly transcriptional (see above), one may expect that its control is accomplished at the same level, and the induction of cell death by either limitation of survival factors or by the appearance of apoptotic factors leads to down-regulation or over-riding of the molecular blockers of cell death initiation. Very little is known about the biochemical nature of these events.

One of the most dramatic developments of the last 2–3 years in cell death research is linking the function of *bcl-2* proto-oncogene to negative regulation of apoptosis. First, it was shown that after introducing *bcl-2* into cells and then cutting off the cytokine supply, the cells survived even though they were supposed to die

[32]. Then, transgenic mice were made bearing the *bcl-2* oncogene in B cells and showed extended survival of these cells [33]. It looks as if *bcl-2* is a kind of antidote to apoptosis and programmed cell death; (i) *bcl-2* protein is topographically restricted to long-lived or proliferating cell zones in tissues that demonstrate apoptotic cell turnover [34], (ii) expression of the human *bcl-2* gene in *C. elegans* reduced the number of programmed cell deaths [35], (iii) over-expression of the *bcl-2* protein in cultured sympathetic neurons prevents apoptosis normally induced by deprivation of nerve growth factor [36], and (iv) the c-*myc*-induced forms of cell death are prolonged when *bcl-2* is over-expressed [22]. At present, nothing is known about the function of the *bcl-2* protein, which has been detected in the mitochondrial membrane and also in the nuclear envelope and endoplasmic reticulum. Of interest is the finding that the first 195 amino acids of *bcl-2* is sufficient for function, provided that a heterologous membrane-associated domain is attached [37]. One recent development that may help clarify *bcl-2*'s function comes from *C. elegans* studies; a gene called *ced-9*, which appears to be the *C. elegans* equivalent of *bcl-2*, has been identified [30]. Its 'gain of function' form of mutant prevents all programmed cell deaths (that is the activity of *ced-3* and *ced-4*), and the loss of the activity of this gene leads to early developmental death of the organism.

There are a number of additional biological and biochemical observations related to prolonged survival of cells. The absence of *bcl-2* from tissues (e.g. the liver) as well as cell types (e.g. endothelial and smooth muscle cells) with regular turnover [34] and the regulated occurrence of *bcl-2*-independent forms of apoptosis (i.e. which occur in the presence of even over-expressed *bcl-2* protein; see next paragraph) suggest the existence of redundant inhibitor elements. Apoptosis has been prevented by down-regulation or, in other types of cell, by activation of protein kinase C [38,39]. It is inhibited by L-acetylcarnitine [40], by tumor promoters [41], through inhibition of the calcineurin (serine/threonine phosphatase 2B) by cyclosporine [42], and after induction of sustained elevation of cytoplasmic calcium [43]. The biochemical target(s) of these effects and their relationship to *bcl-2* expression or function is presently not known.

4. WHAT MAKES NATURAL CELL DEATH SILENT?

A distinctive feature of natural cell death is that despite the dramatic morphological changes (which often include fragmentation and formation of membrane limited particles) the cells are removed without any leakage of intracellular components and without the induction of the inflammatory response and scar formation. The dead cells are recognized and ingested while still intact, protecting tissues from the potentially harmful consequences of exposure to the contents of the dying cell. At least three phagocytosis recognition mechanisms, which do not lead to release of phlogistic agents, such as eicosanoids or cytokines, have been identified in vitro (reviewed in [44]). The use of the $\alpha_v\beta_3$ vitronectin receptor integrin and thrombospondin by a number of macrophages seems to be complemented by receptors recognizing carbohydrate changes and exposed phosphatidylserine on apoptotic cells. The existence of multiple phagocyte recognition mechanisms for apoptotic cells is not unexpected in view of the number of genes involved in removal of cells undergoing programmed cell death in *C. elegans*. Indeed, the observation that the biggest defects in removal of dying cells occur in double mutants (firstly in *ced-2*, *ced-5* or *ced-10* with a second mutation in one of *ced-1*, *ced-6*, *ced-7* or *ced-8*) has prompted the suggestion that there might be distinct, parallel recognition processes which are partially redundant [1].

The swift and efficient engulfment of dead cells is only one of the critical elements which ensure that macromolecules are not released from the dying cells provoking an inflammatory/immune response. Cross-linking of intracellular proteins by the Ca^{2+}-dependent tissue transglutaminase, which is induced and activated in a wide variety of cells undergoing apoptosis or programmed cell death both in vivo and in vitro [3], leads to the formation of a protein shell in apoptotic cells and bodies [45]. Blocking the induction of the enzyme in dying cells by either antisense oligonucleotides or inhibitors results in increased leakage of DNA and protein molecules. Its over-expression, depending upon the intracellular level of the enzyme, leads to increased adhesion to extracellular matrix, higher rate of cell death, apoptotic fragmentation of the cytoplasm and decreased rate of macromolecular leakage during TNF-induced cell death ([46], author's unpublished observations). DNA degradation by DNase I, the physiological epiphenomenon occurring in most apoptotic cells, may also serve to limit release of potentially dangerous genetic material (which may even stimulate production of autoantibodies) spreading from the dying cells. The functional significance of the association between clusterin (*SGPP-2/TRPM-2*) and apoptosis, which occurs in several cell death settings, may be related to tissue protection [47]. Clusterin may provide protection to the dying cells from complement attack or a mechanism to rid the tissues of apoptotic bodies by targetting them into lipoprotein particles.

5. DISTINCT FORMS OF NATURAL CELL DEATH

Based on morphological observations it has been suggested that perhaps all programmed cell death, and even all forms of natural cell death, occurred via apoptosis [2,5]. Detailed examinations and comparison of pro-

grammed cell death in *C. elegans*, in the moth *Manduca sexta*, in neurons and apoptosis of various cell types (thymocytes, hepatocytes, etc.) showed that they differ in terms of cell surface morphology, nuclear ultrastructure, DNA fragmentation, and polyubiquitin gene expression [1,11]. It is also clear, now, that fragmentation of DNA into oligonucleosomes is usually a late event and it does not occur in all forms of programmed cell death or even apoptosis (see above). As molecular characterization of the process has been progressing more and more, data indicate the existence of distinct molecular forms of natural cell death.

There are apoptosis forms, which take place irrespective of the presence of high intracellular levels of *bcl-2* protein. These death forms include the elimination of self-reacting T cells by negative selection [48]. mIgM cross-linking-mediated apoptosis of immature WEHI-231 cells ([49]; an accepted model for elimination of autoreactive B lymphocytes), apoptosis induced by withdrawal of Il-2 or IL-6 [32], by cytotoxic T cells [50] or by TNF or by antibodies raised against the *FAS/APO-1* self-surface protein. Similarly, the programmed death of some *C. elegans* cells (e.g. the male linker cells which fail to die when neighbouring cells are ablated) does not occur by a mechanism that can be inhibited by *ced-9* [1]. These observations suggest that either distinct forms of cell death exist or that the death pathway is accessible at points proximal, as well as distal, to the biochemical function of *bcl-2/ced-9*.

Thymocytes prepared from mice constitutively homozygous for deletion in the *p53* gene are resistant to induction of apoptosis [28,29] by radiation or etoposide (an inhibitor of topoisomerase II). However, they retain normal sensitivity to glucocorticoid or the combination of calcium ionophore and the protein kinase C activator, PMA. The latter reproduces many features of thymocyte death involved in the physiological process of negative selection, which is *bcl-2*-independent [48]. Calcium-dependent activation and glucocorticoid sometimes exert mutually antagonistic effects on thymocyte apoptosis [51], and the regulatory role of c-*myc* exists only in the former [23]. Nuclear proteins *egr-1* and *apt-2* show up in T cells undergoing activation-induced death but not in cells dying after glucocorticoid treatment. These results suggest that the *p53*-independent pathway may itself be very complex, and stimuli which cause apoptosis in thymocytes in the absence of *p53* function may use other transcriptional factors to activate the same set of cell death genes.

Most cell death forms, either dependent or independent of either *bcl-2* or *p53* are delayed or abrogated by inhibitors of RNA and/or protein synthesis. However, it has been also demonstrated in in vitro experiments that apoptosis could proceed or be potentiated in the presence of these compounds, and there are also cases (especially rapidly proliferating cultures of malignant cells) where apoptosis can be directly triggered by the inhibitors [52,53]. Whether these findings reflect distinct forms of apoptosis or variations on a single pathway is not yet known. Interestingly, the *bcl-2*-independent death of target cells induced by cytotoxic T cells does not require de novo protein synthesis [53]. On the other hand, *bcl-2* protects cells from a wide range of non-physiological stimuli, such as azide, colchicine, heat [50], which are independent of protein synthesis. It is also possible that, in some of the cells showing apoptosis in response to protein synthesis inhibitors, the death machinery is already set, but is being actively suppressed by short-living protective proteins [53,54]. Alternatively, some of these adverse effects can be explained by the constitutive expression and drug-induced activation of DNase I: in rapidly proliferating cells the breakdown of the nuclear membrane and the internucleosomal degradation of exposed DNA may lead to the development of apoptosis-like morphology and DNA degradation pattern without the presence and involvement of the complete machinery of natural cell death. In any case, the recognizable and reproducible patterns of gene expression that correlate morphological changes in involutioning tissues and during programmed death-mediated embryonic remodelling [1–4,8,24,41,55] strongly favour the understanding that active gene expression with protein synthesis is an essential part of the natural cell death program in living tissues.

Little attention has been paid to natural cell death forms which produce cells temporarily arrested in one stage of the death program for specific physiological functions. Lens epithelial cells, red blood cells and terminally differentiating keratinocytes undoubtedly represent distinct and specialized forms of natural cell death which, however, are evolutionarily related to, and share common elements with, apoptosis or programmed cell death [3]. For example, the death and cornification of keratinocytes, which is initiated by an unknown signal mechanism and transcriptional factors, is controlled by *bcl-2*, is triggered by calcium influx, leads to nuclear disintegration through internucleosomal DNA degradation, involves transglutaminase-catalyzed cross-linking of proteins to encapsulate cells in a highly cross-linked protein envelope and is regulated by glucocorticoids and retinoids [56].

6. PERSPECTIVES

During the last 2–3 years cell death studies have rapidly moved into the main focus of biomedical research. Genetic and cell biology observations have outlined the major issues, and the obvious clinical as well as pharmacological implications have created almost unprecedented interest. The application of the many tools of biochemistry and molecular biology is just beginning. Functional analysis of gene products related to cell death will lead to understanding, then possibly to phar-

macological control of the process. At present the most challenging tasks are to find the killer genes/proteins, to clarify their biochemical function and to understand how signals are transmitted to and from them. The discovery of novel proteins and/or principles may be needed to link the function of the revealed cell death-related transcriptional factors to effector elements of the cell death process. The comparative and detailed analysis of the many in vivo and in vitro model systems will clarify how many distinct and perhaps redundant or overlapping molecular forms of natural cell death exist, and how much their cell- and tissue-specific manifestations differ.

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