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Death from Inside out: An Overview

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Death from inside out: an overview

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SUMMARY

Although a type of cell death strategically suited to participating in developmental processes has been well known for nearly thirty years, it is only in the recent past that the extraordinary ubiquity of such death has been appreciated. Apoptosis, a term first employed to describe such death defined in structural terms, is associated with a stereotyped set of effector processes, and is driven by genes most of which are familiar as oncogenes or oncosuppressor genes. Dysregulation of apoptosis leads to diseases of enormous social importance such as cancer and AIDS.

1. INTRODUCTION

The papers that follow in this volume deal with various aspects of cell death 'from inside out'. By this, their authors mean that the dying cells they study are contributing in an active way to the triggering and execution of the processes that lead to their own demise. This view of cell death, prevalent among developmental biologists for many decades (Saunders 1966), has taken some time to gain credibility in other branches of biology and pathology. Perhaps this was in part because these disciplines were already familiar with a well-described process (necrosis) characterized by breakdown of cellular energy supply, failure of cellular volume homeostasis, plasma membrane rupture and an ensuing acute inflammatory reaction (Trump & Beresky 1994): events that did not seem congruent with the subtle removal of cells during the sculpting of developing tissues. Perhaps also, even in developmental biology, the cell biological tools were for long not available with which to dissect the mechanisms of death. Within the past four or five years the situation has changed in a scale and at a rate that few could have predicted. A set of genes has been identified that appear to activate or modify a stereotyped programme of effector events, orchestrated to ensure both the rapid demise and practically instantaneous recognition and removal of the dying cell. Moreover, these genes are members of families that are highly conserved between species, suggesting that this process of death may be fundamental to the management of cells and tissues in metazoan organisms. Whereas some of these genes were hitherto unknown, others are extremely familiar in the context of cell proliferation and cancer. A picture is emerging, although not yet completely formed, that death 'from inside out' is a process not too dissimilar in organization to cell division, perhaps using analogous or even identical signalling and effector molecules.

2. MORPHOLOGICAL CONSIDERATIONS

The structural aspects of 'death from inside out' were the first to be resolved, and have now been reviewed many times (Wyllie *et al.* 1980; Arends & Wyllie 1991). Key elements are shrinkage of cell volume, loss of specialized plasma membrane regions such as microvilli, morphological conservation of most cytoplasmic organelles, progressive perinuclear chromatin condensation, and exposure of surface signals that can facilitate engulfment of the dying cell by adjacent phagocytes. This sequence of events was first recognized clearly in hepatocytes *in vivo* (Kerr 1971) and the process underlying them was named apoptosis in 1972, in recognition of its wide significance in tissue homeostasis (Kerr *et al.* 1972).

A corollary of the organization of apoptosis is its inconspicuous nature in tissue sections, even when it is responsible for extensive and rapid cell loss. The time interval between commitment to death and the appearance of the first characteristic cellular features varies according to cell type and lethal stimulus, but there is agreement that the time from first appearance of the structural changes until the dying cell disappears within the phagosome of the ingesting cell, may be a matter of an hour or two, perhaps less. New methods for identification of the changes in the nuclei of apoptotic cells may render them more conspicuous (Gavrieli *et al.* 1992; Ansari *et al.* 1993), but the accurate assessment of the extent of cell death in tissues usually requires scanning of several thousand normal cells, as the proportion of apoptotic cells is often much less than 1%. Because of the short 'washout time' for which apoptotic cells are recognizable, and their total disappearance thereafter, such low percentages can nonetheless be responsible for major reductions in total cell number in a tissue (Howie *et al.* 1994).

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3. EFFECTOR MECHANISMS

Chromatin condensation is associated with evidence of chromatin cleavage, first to fragments corresponding in size (50 and 300 kb) to the loop and rosette domains into which chromatin is organized (Roy *et al.* 1992). Thereafter, many cells show cleavage down to mononucleosome and oligonucleosome size, to engender the familiar 'ladder' pattern on DNA electrophoresis (Wyllie 1980). Attempts to purify the endonucleases concerned have identified a neutral, Mg-dependent nuclease of around 19 kDa that bears immunological similarity if not identity to DNAse I (Gaido & Cidlowski 1991; Peitsch *et al.* 1993). Conversely, in other cell types, this nuclease is not present in measurable quantity, but there is an acid nuclease of *ca.* 35 kDa, that can cleave DNA independent of ambient calcium or magnesium concentration (Barry & Eastman 1993). Several other proteins appear in apoptotic cells, but are less evident or absent in their viable counterparts. It is tempting to assume that they are effector proteins of apoptosis, particularly when their functions seem to be what is necessary to bring about the cellular changes known to occur in apoptosis. At present, however, there are few defined molecular species whose roles as effectors of apoptosis have been stringently established. Thus, apoptotic cells appear to contain a site-specific ribonuclease that cleaves 28*S* rRNA in a non-processive manner (Houge *et al.* 1993). Apoptotic bodies contain proteins that are insoluble in ionic detergent, apparently as a result of cross-linking by a tissue-type transglutaminase (Fesus *et al.* 1991). There are a variety of membrane changes that may permit expression of the signal for phagocytosis (Duvall *et al.* 1985; Savill *et al.* 1990; Fadok *et al.* 1992). Some apoptotic cells express the sulphoprotease clusterin (TRPM-2) on the plasma membrane (Monpetit *et al.* 1986), but this is unlikely to be either necessary or specific for apoptosis. Many cells do not enter apoptosis if RNA and protein synthesis are blocked, but this also is not universal. Protease inhibitors block apoptosis in several cell types (Gorczyca *et al.* 1992), and it is interesting that specific proteases are essential both for the programmed death of cells in the nematode *Caenorhabditis elegans* (Yuan *et al.* 1993) and for the death of the targets of killing by cytotoxic T cells (Shi *et al.* 1992). The lethal agents released from the granules of these cells have long been suspected of including a cocktail of effector molecules required for apoptosis. New, currently anonymous molecular species associated temporally with apoptosis are being intensively searched for by subtractive and differential display techniques.

Despite the fact that many of these effector molecules are incompletely defined, it is already clear that cells differ greatly in their content of some of them. We observed that fibroblast lines contained widely differing activities of endogenous nuclear endonuclease, even although these lines all derived from a common parental stock (Arends *et al.* 1993). Moreover, the nuclear endonuclease activity correlated broadly with the ease with which cells from the different lines underwent apoptosis during log-phase growth in culture. Thus it is possible that cells exist in at least two different states with regard to apoptosis, one in which they are endowed with the necessary effector proteins (we have called this the *primed* state), and would enter the process if suitably *triggered*, and another in which new effectors would need to be synthesized before apoptosis could proceed (the *unprimed* state) (Arends & Wyllie 1991). In these experiments, the various related fibroblast lines were generated from their common parent by independent transfection of a variety of oncogenes. Because unwanted and undetected events might have occurred during the selection of these lines, this experimental design is not adequate to reveal the nature of the genes responsible for the movement into and out of the primed state. It was of interest, nonetheless, that high apoptosis lines consistently resulted from transfections with the human c-*myc* proto-oncogene in constitutive expression vectors.

4. GENETIC REGULATION

In more definitive experiments, candidate genes for regulation of apoptosis have been inserted into cultured cells under control of inducible promoters (Yonish-Rouach *et al.* 1991). Transgenic animals have been constructed in which the genes under test are expressed in a tissue-specific manner (McDonnell *et al.* 1989; Strasser *et al.* 1991). There are now several types of mice constitutively disabled in respect of particular genes, by germ-line mutations introduced through exposure to mutagens (Watanabe-Fukunaga *et al.* 1992) or as a result of engineered homologous recombination events (i.e. 'gene knockout' mice) (Clarke *et al.* 1992, 1993; Lowe *et al.* 1993*a*; Veis *et al.* 1993). These powerful techniques, sometimes applied together, are providing an increasingly detailed picture of the role of familiar oncogenes and oncosuppressor genes in the regulation of cell death.

Many of these developments will be described in later articles, but three generalizations are made here. First, expression of the proto-oncogene c-*myc* renders cells susceptible to apoptosis. This is true of cells of fibroblast (Evan *et al.* 1992; Fanidi *et al.* 1992; Bissonnette *et al.* 1992) and myeloid (Askew *et al.* 1991) lineages, although some lymphoid cell lines show rather different features (Yuh & Thompson 1989). As c-*myc* expression also sustains movement around the cell proliferation cycle, one attractive explanation of this finding is that c-*myc* induces a state in which both cell proliferation and apoptosis become possible, the critical choice between them depending upon additional considerations, such as the availability of growth factors (Evan *et al.* 1992). This dependence upon c-*myc* therefore emphasizes the existence of a high turnover state, in which cell proliferation and cell death are likely to coexist, their relative quantities being determined by the microenvironment. The striking coincidence of proliferation and death within the same areas in many tissues gives credibility to this view.

The second generalization is that activity of certain

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genes protects cells in this high turnover state from apoptosis. The products of many of these genes are *survival factors*; they are not necessarily mitogens, although the surviving cells may of course proliferate if suitable conditions exist. Examples of such survival factors are the *bcl-2* family (Vaux *et al.* 1988) and the tyrosine kinase *abl* (Evans *et al.* 1993), the 55 kDa protein encoded by the adenovirus early region gene E1b (White *et al.* 1992), and the EBV transforming protein LMP-1 (Gregory *et al.* 1991), which may act in part by induction of host cell *bcl-2* (Henderson *et al.* 1991). All these survival factors confer resistance on cells that were previously sensitive to apoptosis. The resistance is pleiotropic in that it applies to a variety of pharmacologically diverse agents. Activity of the oncosuppressor gene *rb-1* also protects tissue cells from apoptosis (Clarke *et al.* 1992), but this may be fundamentally different from the action of survival factors as it is almost certainly not compatible with coexistent cell proliferation. The *rb*-dependent, apoptosis-resistant state may correspond to the growth arrest state of fibroblasts with down-regulated c-*myc*.

The third generalization is that cell injury – and in particular injury that causes DNA double-strand breaks – initiates apoptosis through p53. This was first demonstrated by insertion of a temperature-sensitive mutant of p53 into an IL-3 dependent myeloid cell line (Yonish-Rouach *et al.* 1991). At 37°C, when p53 was expressed in mutant (oncogenic) conformation, the cells proliferated, whereas at 32°C, when p53 was expressed in wild-type conformation, the cells underwent apoptosis. Later, it was shown that this effect of wild-type p53 applies at physiological levels of expression, since cells from animals expressing the normal two copies of wild-type p53 differ in their radiation sensitivity from cells of animals lacking any functional p53 gene (Lowe *et al.* 1993*a*; Clarke *et al.* 1993). Moreover, cells from heterozygotes, in which there is only a single copy of p53 show intermediate effects. Lack of p53 leads to a remarkable, total loss of apoptosis in response to ionizing radiation, even at high doses (14 Gy). This effect is found in many cell lineages: thymic T cells (Lowe *et al.* 1993*a*; Clarke *et al.* 1993), myeloid precursor cells (Lotem & Sachs 1993), intestinal epithelial cells (Merritt *et al.* 1994; Clarke *et al.* 1994) and in results still to be published, lymph node T cells and marrow pre-B cells. Although fibroblasts do not show p53-dependent apoptosis after radiation, their capacity to initiate apoptosis is revealed when they are manipulated to express the adenovirus E1a transforming protein (Lowe *et al.* 1993*b*).

5. HYPOTHESES AND IMPLICATIONS FOR DISEASE

These generalizations lead to new hypotheses relating to the regulation of cell number within tissues. It is probable that most growth factor stimuli reach tissue cells either through contact with other cells (whether homo- or heterotypic) or from the extracellular matrix. By this means local microenvironments are set up in which such stimuli are available, and outside of which they are not. While within the supportive microenvironment, cells are free to survive and proliferate: c-*myc* is expressed and apoptosis is not induced. Cells that find themselves outside, however, may survive in growth arrest, if they down-regulate c-*myc*, or may be deleted by apoptosis if they do not. There is very little information on the factors that might influence the choice between these options. In populations such as those in the lymph node follicle centre during B-cell affinity maturation, it is clear that the 'outside' cells mostly die; in this case they are those that lose out in the competition for engagement with the antigen presented on the follicular dendritic cells (Liu *et al.* 1989). Some cells, however, may be earmarked for survival, even should they leave the supportive microenvironment, by virtue of induction of genes such as *bcl-2*, coding for survival factors. In the follicle centre model, these are the memory B cells. A corollary of this model is that the cells most sensitive to death as a result of DNA damage will be those most equipped for apoptosis and least protected by survival factors. As indicated above, this is indeed the situation in many tissues: the follicle centre, the haemopoietic cells of the bone marrow and the lower third of the intestinal crypt epithelium being outstanding examples.

There are also interesting implications for major disease processes, of which two examples, cancer and AIDS, will be considered here. First, what is the fate of cells that survive, following DNA injury, because they lack functional p53? Animals without functional p53 accumulate such cells in large numbers, even after doses of x-irradiation that cause many strand breaks and mutations. Such animals have a high risk of cancer development, the heterozygotes showing a wide spectrum of primary sites, the homozygotes mostly dying early from T cell thymomas (Purdie *et al.* 1994). In both cases, the tumours are usually aneuploid. It is a reasonable, although still strictly unproven assumption, that the extra cells – those that would have been deleted in a normal animal – are the source of these malignant tumours. In this way, cancer can be conceived of as a disease resulting from deficiency in apoptosis.

The second example is the depletion of T cells in HIV-1 infection. Here the critical lesion appears to be inappropriate deletion, instead of proliferation, of T cells during activation (Laurent-Crawford *et al.* 1991; Banda *et al.* 1992), and perhaps specifically of cells otherwise destined to engender memory cells during repeated T cell-mediated responses (Howie *et al.* 1994). In this way, capacity for specific responses to often-repeated infection is selectively eroded, providing an elegant explanation for the well-known susceptibility of AIDS patients to infection by organisms of low pathogenicity that are commonly present in the human environment.

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