To live or die — a cell’s choice

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Introduction

Living cells face a constant bombardment from their environment. Neighbouring cells, hormonal signals, foreign bodies and nutrients all contribute to the microcosm surrounding the cell, each interacting with the cell surface and many initiating intracellular pathways. The thousands of different proteins expressed produce hundreds of pathways with positive and negative feedback loops. In order for a multicellular organism to function correctly, each cell must efficiently decode, filter and respond to these messages. The correct response is essential to maintain the viability of the whole organism. The accumulation of detrimental signals can lead to cellular injury, after which a cell has four options (Figure 1). These options, which may not be exclusive of one another, are cell proliferation, differentiation, cell-cycle arrest (called senescence if the arrest is permanent) and genetically regulated cell death by apoptosis. (Another form of cell death, necrosis, is considered accidental and not an active choice by the cell.)

In the past few years our knowledge of the above pathways has increased dramatically. This chapter does not discuss these pathways in detail but focuses on what causes a cell to choose a particular pathway, especially apoptosis.

Cell death

Early work proposed a model of cell death that sorted the events into three stages: cell commitment, execution and clearance. In this chapter attention will be given to the first stage only — commitment. This is the crossroads at which...
the cell integrates incoming signals and has to decide what action to take: whether to continue to live and divide, or press the self-destruct button.

After integration into the cell’s vast communication network, most signals will effect only minor alterations in cellular function. Very little may appear to have changed from the outside and it is assumed that life goes on as normal. However, subtle changes may have occurred that are setting in motion the eventual death of the cell.

Let’s start with a nematode worm, Caenorhabditis elegans to be precise. Several genes were identified that are necessary for, and sufficient to cause, cell death. They were named ced-3 and ced-4 (ced stands for Caenorhabditis elegans death). Another gene, ced-9, was found to inhibit ced-3 and ced-4 and is thus able to sustain life [1].

Interestingly, Bcl-2, the human homologue of the nematode CED-9 protein, is partially able to prevent cell death in C. elegans, revealing the level of conservation of the function of the molecule between species [2].
**Inherent properties of the cell**

This same stimulus can produce radically different effects in different cells. For example, thymocytes and lymphoid or myeloid cell lines undergo cell death in response to DNA-damaging ionizing radiation. However, following the same stimulus, fibroblasts respond with cell-cycle arrest [3].

**Cellular context and genetic make-up**

The cellular context of a cell describes the particular genes expressed in the cell and their relative protein levels. Together, these determine cellular function and how it responds to various stimuli. Cellular context is affected by the function of the cell in the body, the stage of differentiation of the cell and the intracellular micro-environment.

In the case of cell fate, the most commonly studied cells are lymphoid, myeloid, thymocyte and fibroblast cell types. Following DNA injury these cells all express p53-induced proteins such as Bax and Bcl-X1, as well as proteins whose expression is independent of p53, including Mcl-1, GADD34 and c-Jun. Even within particular cell types there are differences in response. For example, if death-inducing surface receptors on T-lymphoid cell lines are activated, there is an increase in c-Myc expression and apoptosis results. However, B-cell lymphomas may be protected from apoptosis by c-Myc expression [4].

So which genes decide cellular fate?

**Transcription factors — p53**

Inducible transcription factors such as p53, E2F and c-Myc are vital for processing and amplifying cellular information. They decide cell fate as they interact with each other and recruit other genes by increasing their transcription. The loss of a gene that enables cell survival, or likewise one that prevents cell death, is likely to have disastrous consequences. In approximately half of all human tumours, p53 is mutated. This shows the importance of this gene in preventing disadvantageous proliferation.

p53 is a nuclear phosphoprotein that has been shown to be activated in response to a single-strand break in DNA, hypoxia, oxidative damage and nucleotide or oncogene imbalance [5]. Hypothesized to bind directly to sites of DNA damage or mismatches, it may serve as a damage detector itself or as part of a larger recognition complex that includes the general transcription factor, TFIIH. Importantly, p53 expression and activity are modulated at many levels within the cell. The main control areas are transcription, translation, post-translation (including phosphorylation and acetylation), cellular localization and protein stability [6] (see also Chapter 7 in this volume). Under normal conditions p53 has a short half-life, being rapidly degraded via an autoregulatory ubiquitin-dependent proteasome pathway promoted by the murine double minute clone 2 oncoprotein (MDM2).
When activated, p53 can induce either growth arrest or apoptosis (Figure 1). Both end points can be observed in different cells at different times, so what determines which pathway is chosen? One theory is that p53 is differentially transactivated; in other words, upstream regulators alter the specificity of p53 target genes. Under conditions of little DNA damage, there is only a slight increase in the level of p53 expression and growth arrest is initiated to allow time for repair of the DNA before subsequent proliferation. In the presence of large amounts of DNA damage p53 is highly expressed and cell death becomes inevitable. Another theory is that altering the cellular location of p53 determines the outcome, as it is translocated during degradation. Indeed, altering the expression levels of MDM2 would provide another means of control.

**Cell cycle and the proto-oncogenes — E2F and c-Myc**

The stage of the cell cycle can predetermine a cell's response to transcription factors. According to Hengstschlager et al. [7], Rat-1 cells in S phase are resistant to cell death induced by activated E2F or c-Myc. This resistance is specific, as the S phase cells were not resistant to death induced by treatment with actinomycin D. As yet the protective mechanism of S phase cells is unknown. Cells in G₁ and G₂ were susceptible to death from c-Myc expression, whereas E2F caused death specifically in G₁. This reveals the importance of the cell cycle in determining cell fate.

E2F is a growth-promoting transcription factor. In the E2F family there are five subsets — E2F-1–5. During times of cellular rest, E2F is bound by one of the retinoblastoma (Rb) family of phosphoproteins. E2F-1, E2F-2 and E2F-3 bind to Rb-1, E2F-4 to p107, and E2F-5 to p130 (see Chapter 7 by H.M. Chan et al.). In its active, unbound form E2F will drive the cell cycle through G₁ to S phase and DNA replication. By maintaining a high proportion of bound E2F, the cell can control cell-cycle progression.

Shan and Lee [8] reported that deregulation of E2F in the presence of p53 causes early entry into S phase and subsequent apoptosis in Rat-2 fibroblasts. Similarly, when the Rb-1 gene is knocked out, leaving E2F in its free, active form, mice are non-viable, with massive cell death from inappropriate S-phase entry and subsequent apoptosis [9]. Interestingly, proliferation may help to rescue cells from otherwise fatal signals. Spyridopolous et al. [10] reported that overexpression of E2F exerts a survival effect in proliferating endothelial cells and restores cell-cycle progression. It seems that inappropriate levels of unbound E2F force S-phase progression, which, if combined with p53 growth arrest signals, may confuse the system into inducing apoptosis. However, when cells are already dividing, overexpressed E2F may simply enhance proliferation. Thus the effect of E2F-1 is in balance with other competing stimuli in the cell cycle. Tumours occur when one or more factors are disrupted, as is the case when E2F-1 is removed in a knockout mouse. Surprisingly, hypoproliferation is not observed; instead we see apparently normal development with later
occurrence of lymphomas, lung and reproductive tract tumours [11], demonstrating how important balance is in the cell cycle.

At the C-terminus of c-Myc are two nuclear-localization domains. The middle DNA-binding portion contains a leucine zipper, a helix–loop–helix motif and a group of basic amino acids used for DNA recognition. The N-terminus contains a transcriptional activation domain. This proto-oncogene is expressed in almost all proliferating cells, but is down-regulated in terminally differentiated cells. It has been shown to promote both proliferation and apoptosis. Its apoptotic action is p53-dependent in some cells but not in others [12]. As with E2F, overexpression of c-Myc in serum-deprived Rat-1 fibroblasts resulted in apoptosis in those with wild-type p53 expression [13], again illustrating the danger of conflicting cellular signals. Interestingly, overexpression of c-Myc also increased expression of the pro-apoptotic protein Bax [14]. It is possible that c-Myc may induce both death and growth pathways upon activation, with active inhibition of death being required for continued cell growth.

**Expression level — Bcl-2:Bax ratio**

Transcription and translation are not the only ways of controlling protein expression. Rapid degradation, conformational changes and proteolytic activation of inactive zymogens can all alter an enzyme’s activity. Protein phosphorylation by kinases and translocation between intracellular compartments are other mechanisms of control.

**Figure 2. The Bcl-2 family**

These cellular proteins regulate cellular fate through their interactions with family members and other cellular proteins such as Apaf-1 [15]. They share varying numbers of (BH) domains, and many contain transmembrane domains (TM) that anchor the protein to intracellular membranes. Here they interact with each other to form dimers; the particular configuration of the dimer is variable, with both homodimers and heterodimers being formed. Some of the BH3-only proteins are not bound to membranes and are thought to translocate to the membrane following activation.
Maintaining strict control over the life and death processes is of obvious importance to the cell. Two proteins from the same family, Bcl-2 and Bax, form a cellular ‘rheostat’ determining which pathway is chosen, i.e., whether the cell lives or dies, respectively. The cell survival protein Bcl-2 contains four conserved domains named BH1–BH4 (Bcl-2 homology domains) (Figure 2) and a hydrophobic C-terminal domain used for insertion into mitochondrial and endoplasmic reticulum (ER) membranes. Anti-apoptotic Bcl-2 and Bcl-XL comprise only one of the three subsets in the Bcl-2 family. Pro-apoptotic proteins, Bax and Bid, and the BH3-only class (containing Bik, DP5, Bim/Bad and Blk) make up the other two.

Studies of the tertiary structure of Bcl-XL have revealed a structure very similar to the pore-forming domain of diphtheria toxin. Bcl-XL contains a pair of core hydrophobic helices that penetrate the lipid membrane and are shielded from the aqueous environment by five to seven amphipathic helices [15]. This is thought to be the key to their function, as they are known to be present in mitochondrial membranes, and the mitochondria play a central role in apoptosis (Figure 3).

**Figure 3. Receptor-mediated and chemically-induced death**
Extra-cellular signals can be classified as either (a) ligands that bind to receptors or (b) chemicals that act directly on the cellular machinery. Ligands, such as Fas or TNF-a, bind to membrane receptors to activate intracellular pathways that eventually lead to cell death. Cytotoxic chemicals bypass the membrane and induce the caspase cascade by acting directly on mitochondria. The end result is the same however, with activation of the effector caspases and the induction of apoptosis. TNFR, TNF-a receptor; TRADD, TNFR-associated death domain protein.
The different members of the family have the ability to form heterodimers with each other. Bcl-2 (pro-survival) competes with Bax (pro-apoptotic) to prevent it forming death-inducing Bax:Bax homodimers. The Bcl-2:Bax ratio is therefore critical in determining sensitivity to apoptosis and eventual cell fate. Factors that alter this ratio in MIN6 and RINm5F pancreatic β-cell lines include serum deprivation and Ca²⁺ chelators such as EGTA and BAPTA [bis-(o-aminophenoxy)ethane-N,N,N’’N’-tetra-acetic acid] [16]. Fatal exposure of MIN6 cells to 4 mM BAPTA or EGTA resulted in a steady decrease in Bcl-2 mRNA levels, whereas Bax mRNA increased in the 24 h following exposure. Removal of serum resulted in a decrease in Bcl-2 levels after 96 h, while Bax protein remained relatively constant. Further work showed that the changes in mRNA corresponded with changes in protein levels.

Environmental influences

Death induced by survival factor withdrawal or exposure to cytotoxic chemicals

Current research suggests that exposure to cytotoxic chemicals or serum withdrawal induces cell death by bypassing the cell’s decision-making process and acting directly on mitochondria to release cytochrome c (Figure 3). This leads to the activation of apoptotic protease activating factor 1 and also caspase 3, the final executioner of cell death.

Hydrogen peroxide (H₂O₂) was used by Bladier et al. [17] to induce both apoptosis and senescence in primary human diploid fibroblasts, the outcome depending on the concentration of the H₂O₂. At 50–100 μM H₂O₂ there was an increased number of cells in G₁ and G₀ and a decreased rate of proliferation. This senescence-like state correlated with an enlarged and flattened morphology. Apoptosis was observed when the H₂O₂ concentration was increased to 300–400 μM. These results indicate a relationship between the concentration of H₂O₂ and the extent of the cellular damage. Interestingly, 200 μM H₂O₂ produced features of both apoptosis and senescence, suggesting that there is a linear relationship between H₂O₂ concentration and cell damage.

Receptor-initiated death

Receptor-initiated death is mediated by the tumour necrosis factor (TNF) family of cell-surface receptors, which are activated after exposure to Fas ligand or TNF-α (Figure 3). Binding of Fas leads to the activation of Fas-associated death domain (FADD) that in turn leads to caspase activation, as indicated by the ability of caspase inhibitors to prevent cell death in response to Fas. However, these inhibitors are ineffective against other forms of apoptotic stimuli such as DNA damage, activated oncogenes or Bak [18].

The intracellular pathways of chemically-induced and receptor-mediated death were studied by Sun et al. [19]. They used TNF-α and Fas to activate receptor-mediated pathways and etoposide to activate chemically-induced
death. The caspase inhibitor, Z-VAD-FMK (benzyloxy carbonyl-Val-Ala-D,L-Asp-fluoromethylketone), was utilized to determine whether any divergence could be found between the two pathways.

The results were intriguing. In receptor-mediated death the inhibitor prevented the biochemical features of cell death, such as cytochrome \( c \) release, caspase 8 activation and cleavage of Bid. Sun et al. revealed an action upstream of the final commitment to die. In chemically-induced death the inhibitor again prevented, death but not cytochrome \( c \) release or the cleavage of Bid. Sun et al. took this to indicate that the inhibitor was acting at the level of preventing the manifestation of death (by acting on caspase 9) but not the decision to die, as indicated by the presence of cytochrome \( c \) and Bid. They concluded that caspases have a varying influence on the outcome of death depending on the type of stimulus. In receptor-mediated death these enzymes are inherent to the decision-making process, while chemically-induced death sees them relegated to the level of executioners of death only. This experiment strongly suggests that the method of inducing death is critical to whether the decision-making process is utilized or overridden. Viruses are a classic example of bypassing the intracellular death decision process.

**Viruses — overriding cell choice**

To survive inside a living cell without the cell recognizing the invasion and eliminating itself, a virus must block the cell death pathway. In this way, the virus overrides the decisions made by the cell to set its own agenda. There are three main cellular control points which viruses attack. Adenovirus and Epstein–Barr virus produce gene products E1B19K and BHRF1, respectively, which mimic the cell survival functions of Bcl-2. This transforms cells into continuously proliferating cells unable to induce death. Simian virus 40 and human papilloma virus modify p53 action and prevent its tumour-suppressor action, thus allowing dysregulated cellular proliferation. Cowpox and insect baculovirus produce CrmA and p35, which act as caspase inhibitors and prevent the execution of cell death.

**The mechanism of death**

The appearance of phosphatidylserine on the outside of the plasma membrane was assumed to indicate commitment to cell death. It may facilitate recognition by phagocytes, subsequent phagocytosis and degradation of the apoptotic cell. This can be visualized by the binding of fluorescein-labelled annexin V.

Hammill et al. [20] cross-linked B-cell lymphoma membrane immunoglobulin receptors to initiate cell death. They found that, although many cells bound annexin V, they were viable and could resume normal growth once the cross-linking was reversed. This demonstrates that the commitment to die had not yet taken place. Therefore, we can postulate that in
these cells the timing of the critical decision is after annexin V binding but before caspase activation.

**Caspases**

Execution is carried out by activation of cysteinyl-aspartyl proteases, or caspases. Caspases are synthesized as inactive precursors and are activated via proteolytic cleavage by apoptotic protease-activating factor-1 (Apaf-1), FADD or other caspases (Figure 3). The active complex is a tetrameric enzyme made of two heterodimer caspase molecules.

Caspases 8 and 9, the initiator caspases, are interleukin-converting-enzyme-like proteases that are able to initiate the death cascade through recruiting other caspases. Caspase 8 is believed to be one of the first caspases cleaved by FADD following Fas activation. Once active it will activate other procaspase 8 as well as caspases 3, 6, and 7, thus creating a death cascade. The other initiator caspase, caspase 9, also targets these effector caspases, but in response to activation by Apaf-1. Harvey et al. [21] reported that activation of these caspases alone is necessary, but not sufficient, to cause death. They reported that Bcl-2 is able to rescue cells after activation of initiator caspases, but not once effector caspases had been activated. Therefore it is assumed that Bcl-2 acts at the boundary between initiator and effector caspases to prevent death.

Once the effector caspases are activated death is certain. There is an irreversible drive towards death that leads to DNA fragmentation, nuclear condensation, cytoskeletal collapse, genome degradation, formation of apoptotic bodies and cellular disintegration (Figure 4) [22].

![Figure 4. An apoptotic islet β cell with nuclear condensation and apoptotic bodies visualized using haematoxylin and eosin staining](image)
Mitochondrial permeability transition pore (MPTP)

Apart from leading to a caspase death cascade, activation of the initiator caspases also opens the MPTP via cleavage of the Bcl-2 homologue, Bid, by caspase 8. Alternatively, the MPTP can be opened via a Bid-independent pathway following exposure to cytotoxic chemicals. Opening of the pore leads to further amplification of the cascade via release of cytochrome c and subsequent activation of Apaf-1 (Figure 3) [23].

Green and Amarante-Mendes [24] suggested that the opening of the MPTP is one of the irreversible points in the pathway leading to cell death. In addition, Scorrano et al. [25] have shown that the opening of the pore precedes, and is causally related to, apoptosis.

The pores are located in the mitochondrial wall at points where the inner and outer membranes make contact. Opening the pore directly by treatment with the cytotoxic chemicals oligomycin and atractyloside [24] leads primarily to dissipation of the proton gradient and uncoupling of the respiratory chain. Secondary events include generation of reactive oxygen species and cessation of ATP production. Apoptosis-inducing factor is released along with cytochrome c and contributes to the dramatic final stages of apoptosis. The entry of water through the open pore leads to mitochondrial swelling and rupture. There is some controversy over whether cytochrome c causes MPTP opening or is simply released as a result of it.

Future perspectives

Cell pathway choice is best viewed as an iterative process. Each injury or signal provokes a response that channels the cell in a particular direction. In the cell the presence or absence of control genes like p53, E2F or c-myc has a great influence on whether the next step is undertaken. Their relative expression levels in the cell vary over time and influence the likelihood of progression to the next step in the pathway. The effective manipulation of these control genes provides an entrance into the very control centre of life. Our increasing ability to regulate life and death of chosen cells opens up the possibility of new therapies in the fight against many diseases.

Discovering what causes a cell to choose to die is especially important for type 2 diabetes where there is a loss of functional β-cells within the diabetic pancreas. Under conditions of cellular stress caused by hyperglycaemia and hyperlipidaemia some people become diabetic while others do not. Elucidating the mechanism behind this selective β-cell death is one of the key areas for future research. Conversely, it is important to find out what causes a cell to become a tumour cell and choose continuous proliferation rather than death.

Much research is focusing on the exact point at which cell death becomes inevitable and the process irreversible. The same cell may well use different mechanisms in response to various stimuli. Alternatively, different cell types utilizing different components may use the same mechanism. At present the
opening of the MPTP, the activation of effector caspases and the ratio of Bcl-2 to Bax all appear to be key points of cell commitment.

Summary

- Cell death is one of several choices a cell faces in response to injury. The cell's inherent properties and its external environment determine which pathway is chosen.
- Interaction between transcription factors such as p53, E2F and c-Myc acts to finely tune the pathway selection process.
- Once the cell death pathway is initiated, survival proteins can stop it at different stages upstream of the activation of effector caspases.
- The exact point of no return along the cell death pathway is unknown, but is likely to vary between cells. It is unlikely to be a single step, but rather a short process leading to irreversibility of the pathway.

References


