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Non-lethal outcomes of engaging regulated cell death pathways in cancer

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Regulated cell death (RCD) is essential for successful systemic cancer therapy. Yet, the engagement of RCD pathways does not inevitably result in cell death. Instead, RCD pathways can take part in diverse biological processes if the cells survive. Consequently, these surviving cells, for which we propose the term 'flatliners', harbor important functions. These evolutionarily conserved responses can be exploited by cancer cells to promote their own survival and growth, with challenges and opportunities for cancer therapy.

1

The term RCD, occurring as a result of a molecular pathway, has replaced 'programmed cell death', as the latter was specifically coined to refer to cell death that occurs at defined times during development[1]. RCD shapes the physiological development of tissues and organs, maintains homeostasis and plays various roles in multiple disease processes[2,3]. The molecular machineries of RCD can be initiated by diverse mechanical, biological, physical and chemical stresses and are influenced by various cell-intrinsic and -extrinsic signals. This is opposed to 'accidental' cell death, which is an immediate fatal response to severe physical, mechanical or chemical damage and is often referred to as 'necrosis' (ref. 4). The molecular events of RCD pathways and the resulting phenotypic changes have been related to various clinical outcomes, particularly in the context of cancer.

2

While evading cell death has been identified as a hallmark of cancer[5], this is often misinterpreted as suggesting that cancer cells are resistant to RCD. This is incorrect, as highlighted by studies showing that cancer cells can be 'primed for death', and the response to conventional therapy correlates with such priming[6]. It is therefore understood that a hallmark of cancer is an ability of the cell to evade those RCD mechanisms that are engaged to suppress the oncogenic process, not necessarily RCD in general.

The engagement of RCD does not inevitably result in cell death. Cells that survive the activation of an RCD pathway can undergo changes that influence their behavior and/or that of surrounding cells. These may include genomic instability leading to high mutational burden[7,8] and protumor or anti-tumor immune responses[9]. Moreover, preclinical research provides evidence that sublethal engagement of RCD leads to phenotypic adaptations including epithelial–mesenchymal transition and altered interaction within the cell's microenvironment[10–13]. Consequently, sublethal engagement of cell death contributes to metastasis, invasiveness and therapy unresponsiveness but can also present vulnerabilities that might be harnessed for cancer treatment[13–16].

3

Here, we introduce the term 'flatliner' to represent a cell that has engaged a core RCD mechanism but manages to survive, in analogy to a patient who 'flatlines' but is resuscitated. For cells, this is distinct from resistance to or evasion from signals that normally induce cell death. We propose that engaged cell death pathways do not always lead to cell death, and flatliners that survive may have altered properties. We elaborate how cancer cells resist therapy, with particular focus on the molecular mechanisms that underlie the evasion of cell death following activation of an RCD pathway, and how survival of flatliners may account for phenomena associated with cancer persistence. Furthermore, we assess how our knowledge and recent advances in the cell death field translate into our understanding of cancer progression and relapse and how this may uncover new therapeutic opportunities.

4

Finally, we discuss the potential relationship of flatliners to drug-tolerant persister cells, characterized as cancer cells without resistance-associated mutations that survive treatment[14,17]. These definitions are distinct: while both processes are transient (cells revert to the parental drug sensitivity over time), flatliners have demonstrably engaged a core cell death pathway and survived, and in contrast persister cells are defined only by their transient drug-tolerant state. Although we argue that, at least in some cases, engagement of core cell

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death machinery can induce the persister cell phenotype, the distinct definitions of each are important.

RCD pathways in cancer

In the following section, we briefly survey four common RCD pathways: apoptosis, necroptosis, pyroptosis and ferroptosis, and discuss the molecular mechanisms that underlie their evasion.

Apoptosis

Apoptosis refers to cell death associated with the activation of cysteine–aspartate proteases (caspases) mediating cleavage of target proteins, leading to fragmentation of cellular DNA, nuclear condensation, membrane blebbing and rapid clearance before loss of plasma membrane integrity[18]. The biochemical and morphological changes associated with apoptosis are orchestrated by the activity of the executioner caspases, caspase-3 and caspase-7, which cleave hundreds of substrates, including those responsible for the changes mentioned above[19,20]. For example, internucleosomal double-strand DNA breaks during apoptosis are caused by the caspase-activated nuclease (CAD). Another protein, inhibitor of CAD (iCAD), acts as a chaperone to bind and inhibit CAD while folding it into an active nuclease. The executioner caspases cleave iCAD, allowing the active CAD to cause DNA fragmentation[21].

Executioner caspases are activated by initiator caspases (for example, caspase-8 and caspase-9), which cleave the inactive, dimeric executioners to activate them. The initiator caspases are not activated by cleavage but instead by binding and oligomerization of the inactive monomers on activated adaptor proteins. These adaptors and initiator caspases define the apoptotic pathways (Fig. 1).

In the mitochondrial or intrinsic pathway of apoptosis, the adaptor is apoptotic protease-activating factor 1 (APAF1), which binds and thereby activates the initiator caspase (caspase-9), which in turn cleaves and thereby activates the executioner caspases[19]. The latter are inhibited, however, by X-linked inhibitor of apoptosis (XIAP). The activation of APAF1 occurs following mitochondrial outer membrane permeabilization (MOMP), releasing cytochrome c from the mitochondrial intermembrane space, which induces the activation and oligomerization of APAF1. In addition, proteins that interfere with XIAP are also released upon MOMP, derepressing the executioner caspases and allowing apoptosis to proceed[22]. Extensive MOMP in a cell can result in a mitochondrial energetic catastrophe that usually ends in cell death, even if executioner caspase activation is insufficient[23].

MOMP is caused by the action of the pro-apoptotic effectors of the B cell lymphoma 2 (BCL-2) family, for example, BAX and BAK. These are antagonized by anti-apoptotic BCL-2 proteins, for example, BCL-2, BCL-xL and MCL-1. A third type of BCL-2 proteins, trihydrobiopterin (BH3)-only proteins, function to inhibit the anti-apoptotic proteins and/or activate the pro-apoptotic effectors. The functions of the BCL-2 proteins have been reviewed elsewhere[24]. Engagement of apoptosis in cancer cells is triggered by many chemotherapeutic drugs and radiation therapies, which results in the activation of BAX and BAK through increased function of BH3-only proteins and decreased anti-apoptotic BCL-2 protein function[25]. Cells that are poised to undergo MOMP ('primed for death') are associated with better prognosis in response to conventional therapy[6], and dynamic BH3 profiling, in which drugs are tested for their ability to prime cells for induction of MOMP by BH3 peptides, has shown promise in predicting therapeutic efficacy[26].

One way for cancer cells to survive in the face of chemotherapeutic insult is to prevent engagement of cell death pathways via mutation or other mechanisms that permanently disrupt the action of the drug, referred to as resistance. This is distinct from survival following engagement of such a pathway and is not considered further here. Previously, MOMP was widely considered a 'point of no return' for cells, but we now know that cells can survive a degree of MOMP and sublethal caspase activation[16,27] (Fig. 2). It has been observed that not all mitochondria necessarily release cytochrome c upon stimulation with chemotherapeutic agents (incomplete MOMP; iMOMP). iMOMP allows for repopulation of cells with healthy mitochondria and increased clonogenic survival once the death-inducing stress is removed. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) overexpression has also been shown to promote clonogenic survival in cells displaying iMOMP, promoting increased glycolysis and a transient increase in mitochondrial mass[28]. In another study, the repair of double-strand DNA breaks induced upon caspase-mediated activation of CAD was noted as a requirement for cancer cell survival[8]. Widespread MOMP, followed by extensive apoptotic caspase activation, is normally lethal, but cells that engage the mitochondrial apoptosis pathway can survive.

A second pathway of apoptosis involves activation of the initiator caspase, caspase-8, by its adaptor FADD. This can occur upon ligation of death receptors of the tumor necrosis factor (TNF) receptor (TNFR) family, for example, TNFR1, FAS (CD95) and TRAIL receptors, and is often referred to as the death receptor or extrinsic pathway of apoptosis (although other, intrinsic mechanisms, including non-death receptor processes, exist to activate FADD–caspase-8 (ref. 29)). Active caspase-8 cleaves and thereby activates the executioner caspases.

Flice-like inhibitor of apoptosis, $cFLIP_L$ (herein, FLIP), resembles caspase-8 but lacks a catalytic cysteine. If FLIP is present, it binds to a monomer of FADD-bound caspase-8, preventing oligomerization of the latter, and thus prevents apoptosis[30]. However, the caspase-8–FLIP heterodimer is proteolytically active and performs other functions, as discussed below. The extent to which caspase-8 and death receptor signaling contribute to cancer is not well understood.

The death receptor pathway of apoptosis appears to be an important mechanism for anti-tumor immunity, as cytotoxic lymphocytes deploy death receptor ligands (FAS or CD95, TRAIL) as one way to kill cancer cells. Caspase-8 is mutated or silenced in some cancers[31]. However, whether this represents an immune-evasion mechanism or an escape from a tumor-suppressor mechanism is not clear. Intriguingly, most cancers express FAS[32] and many express receptors for TRAIL[33], suggesting that such receptors may have roles beyond cell death that are important in cancer maintenance[34].

Cells that activate the death receptor pathway can survive, suggesting that low levels of executioner caspase activation are tolerated[35]. Survival following engagement of apoptosis and activation of executioner caspases has been termed anastasis (defined as cell survival despite activation of executioner caspases)[36]. Using a fluorescent marker of caspase-mediated cleavage, studies in flies indicated that many cells in the developing animals display evidence of caspase activation without apparent cell death[37]. Studies in primary and transformed mammalian cells revealed features of anastasis following induction of apoptosis, including DNA damage, oncogenic transformation and induced gene signatures[36]. Although MOMP was not assessed in these studies, evidence (discussed above) strongly suggests that cells can survive MOMP and thus might be considered to have undergone anastasis[15,16].

Necroptosis

While necrosis often refers to uncontrolled cell death, we now recognize that there are regulated forms of necrosis. Among these is necroptosis[38], in which receptor-interacting kinase (RIPK)3 phosphorylates mixed-lineage kinase-like (MLKL) protein (Fig. 1), which then oligomerizes and is incorporates into the cell membrane, forming a large pore and inducing necroptosis. Three proteins are known to bind and thereby activate RIPK3: RIPK1, TIR-domain-containing adaptor inducing interferon-β (TRIF), and Z-DNA-binding protein 1 (ZBP1)[39]. These, in turn, are activated by ligation of death receptors, some Toll-like receptors and interferon (IFN) receptors, respectively. RIPK1, however, associates not only with RIPK3 but also with FADD and, in the absence of FLIP, can cause apoptosis via activation of caspase-8 (ref. 40). If FLIP is present, apoptosis is blocked, while the catalytic activity of FADD–caspase-8–FLIP cleaves RIPK1 and the associated

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