Heterogeneity of Cell Death

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Cancer evolution · Cell death heterogeneity · Chromosome fragmentation · Genome chaos · Genome theory · Mitotic cell death · Non-clonal chromosomal aberration

Abstract

Cell death constitutes a number of heterogeneous processes. Despite the dynamic nature of cell death, studies of cell death have primarily focused on apoptosis, and cell death has often been viewed as static events occurring in linear pathways. In this article we review cell death heterogeneity with specific focus on 4 aspects of cell death: the type of cell death; how it is induced; its mechanism(s); the results of cell death, and the implications of cell death heterogeneity for both basic and clinical research. This specifically reveals that cell death occurs in multiple overlapping forms that simultaneously occur within a population. Network and pathway heterogeneity in cell death is also discussed. Failure to integrate cell death heterogeneity within analyses can lead to inaccurate predictions of the amount of cell death that takes place in a tumor. Similarly, many molecular methods employed in cell death studies homogenize a population removing heterogeneity between individual cells and can be deceiving. Finally, and most importantly, cell death heterogeneity is linked to the formation of new genome systems through induction of aneuploidy and genome chaos (rapid genome reorganization).

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Cell death is a diverse and complex group of processes occurring in a number of forms that is ultimately caused by stress to the cellular system [Stevens et al., 2011a; Galluzzi et al., 2012]. Of these types of cell death, apoptosis is the most studied, as it has been the primary focus of cell death research for decades. Measures of apoptosis, however, are inadequate at estimating the amount of cell death that occurs [Hendry and West, 1997]. For example, in cancer, apoptosis does not always faithfully represent the amount of cell death occurring in a tumor [Kondo, 1995; Erenpreisa and Cragg, 2001; Abend, 2003]. It has been suggested that mitotic cell death (MCD) represents the major form of cell death that occurs in treated tumors with autophagy and necrosis also playing a role in the reduction of tumor mass [Heng et al., 2004; Stevens et al., 2004, 2007, 2011a]. Thus, more than one type of cell death occurs in the same tissue at the same time.

Cells within a population exhibit heterogeneous behaviors especially in disease conditions, and cell death is not a unique property in this regard [Heppner, 1984; Hendry and West, 1997; Davidoff, 2009; Heng et al., 2009; Stevens et al., 2011b; Heng, 2013b]. This heterogeneity occurs at both biochemical and morphological levels. For instance, similar cell death morphologies can be produced by different biochemical pathways [de Bruin and Medema, 2008; Galluzzi et al., 2012]. The type(s) of cell death that occur rely on a complex interplay between the factors that induce the cell death, cellular status (such as

genome stability) and the availability of proteins, complexes, organelles, and other factors required for a given cell death [Hendry and West, 1997; Calzone et al., 2010; Stevens et al., 2011a]. Cell death also does not occur at the same rate in individual cells [Goldstein et al., 2000a, b; Tyas et al., 2000]. Thus, cell death can be unpredictable at times (e.g. during chemotherapy), while at other times it occurs in a predictable manner (e.g. during *Caenorhabditis elegans* development) [Lettre and Hengartner, 2006; Domingos and Steller, 2007]. On top of this, heterogeneity occurs within normal cellular populations and populations in diseased tissue exhibit increased heterogeneity. For instance, genetic/genomic heterogeneity is ubiquitous in cancer [Heng, 2007; Heng et al., 2011a]. This genetic heterogeneity enhances functional heterogeneity of cells in a population.

In addition to cell death occurring in multiple forms, the outcomes of cell death can also be heterogeneous [Erenpreisa and Cragg, 2001; Stevens et al., 2011a]. Cell death does not always occur with complete fidelity. For a multitude of reasons the execution of cell death can be interfered with, leading to cells with varying degrees of damage, including DNA damage. This damage then can become fixed in the form of mutations, copy number variations or gross chromosomal alterations which can be inherited by progeny. Not surprisingly, escape from cell death due to drug treatment is directly attributable to population variability in protein expression, not a specific pattern of expression in cells [Cohen et al., 2008]. Cell death heterogeneity lies at the heart of one of the key challenges for cancer therapy, drug resistance [Heng et al., 2010a; Stevens et al., 2011a]. Cells that are resistant to the form(s) of cell death induced by a specific drug can quickly become the dominant cell type in a tumor if all other cells are effectively eliminated. Furthermore, the stress of drug treatment can spur adaptive genome evolution such as genome chaos, increasing the likelihood for survivors [Heng et al., 2006b, 2011a, b]. Thus not all cell death-related processes result in a favorable outcome in treating cancer. Increasing our understanding of cell death processes and their inherent heterogeneity will improve our ability to systematically treat multiple diseases, especially cancer.

Multiple Types of Cell Death

In order to fully appreciate the heterogeneity of cell death, some background on the various forms of cell death is required.

Apoptosis

Apoptosis has been the traditional focus of much of the cell death field since its discovery in 1972 [Kerr et al., 1972]. During this time apoptosis has been shown to be a complex process that can occur via multiple pathways in response to multiple stimuli. Prototypical apoptosis occurs in a mitochondrial-dependent manner through activation of caspase proteases which form a cascade and ultimately result in the degradation of nuclear material. Cellular contents are then packaged into blebs that can then be taken up and destroyed by neighboring cells. However, a multitude of apoptotic pathways have been uncovered including intrinsic pathways that are responsive to signals within the cell such as DNA damage or reactive oxygen species (ROS) exposure and extrinsic pathways instituted by cell-cell interactions. Furthermore, apoptosis occurs both in caspase-dependent and independent manners and similarly can be dependent or not on mitochondrial function [Galluzzi et al., 2012].

Necrosis

Necrosis occurs both as an unregulated and a regulated form of cell death due to overwhelming stress including drug toxicity, ROS exposure or mechanical damage or as a regulated form of cell death upon certain conditions. Regulated necrosis can occur in response to a number of factors, especially if the caspase system is compromised. The RIP family of proteins is often used as a marker of regulated necrosis [Galluzzi et al., 2009; Vandenabeele et al., 2010].

Autophagy

Autophagy is the process of self digestion which usually involves extensive vacuolization of the cytoplasm of the cell [Galluzzi et al., 2008b]. During the process, contents of the cell are isolated into phagosomes which fuse with lysosomes to form autophagosomes and digest the contents therein. Autophagy is primarily used to recycle organelles and large protein complexes and serves as a central system for adaptation to stress [Galluzzi et al., 2008b]; however, large scale autophagy can lead to the elimination of entire cells [Eisenberg-Lerner and Kimchi, 2009]. Like apoptosis, reduction of the ability to undergo autophagy increases the risk of cancer [Qu et al., 2003].

Mitotic Cell Death

Mitotic cell death (MCD) is the most common cell death occurring in tumors [Roninson et al., 2001]. MCD has been largely described on a morphological basis, though some progress has been made in identifying cel-

lular and molecular mechanisms of MCD. Cells undergoing MCD often show nuclear aberrations such as multi- and micronuclei. MCD occurs in 2 major forms, one occurring directly during mitosis (chromosome fragmentation or C-Frag), and one following a failed mitosis (mitotic catastrophe). Each of these types of MCD typically shares a prolonged mitotic arrest.

Mitotic catastrophe is a form of MCD that primarily occurs after a failed mitosis and involves some of the apoptotic machinery including caspase 2 activation [Castedo et al., 2004b]. TP53 and other TP53 family members have also been implicated with mitotic catastrophe in some cases [Nitta et al., 2004; Taylor et al., 2006]. TP53 and family members have been shown to impact mTOR (mammalian target of rapamycin) signaling and elicit senescence [Roninson et al., 2001].

C-Frag is another form of MCD that occurs directly during mitosis (fig. 1) [Stevens et al., 2004, 2007; Ye et al., 2007]. It is detectable in cytogenetic preparations, and it is identifiable by its unique morphology of degraded, condensed chromosomes and loss of cell membrane integrity [Heng et al., 2004; Stevens et al., 2007]. C-Frag is distinct from caspase-reliant apoptosis as it is not inhibited by broad spectrum caspase inhibitors or by overexpression of Bcl-2. PARP is also rapidly degraded during C-Frag, separating it from some forms of regulated necrosis [Stevens et al., 2011a]. In the past, C-Frag has been confused with premature chromosome condensation; however, these 2 phenomena are distinct [Stevens et al., 2007, 2010].

Many Types of Cell Death Coexist

Each type of cell death is not exclusive, different types of cell deaths often occur within the same cellular population [Abend, 2003]. Apoptosis and necrosis are often shown to occur as variable responses in a cell population to certain treatments [Xiao et al., 2002]. Similarly, apoptosis and autophagy can occur within the same population [Gonzalez Polo et al., 2005]. It has been argued whether these cell deaths are complementary or in opposition to each other. Autophagy (though not necessarily death by autophagy) occurs in response to stresses such as starvation and has been proposed to be a method of cellular adaptation. Autophagic death, however, can be induced or can occur if defects exist in apoptotic pathways [Galluzzi et al., 2008b, 2012]. It is therefore reasonable that co-occurrence of autophagy and apoptosis is both adaptive and a method to eliminate cells. C-Frag and apoptosis occur within the same populations of cancer cells with higher drug concentrations favoring apoptosis [Stevens et al.,

Fig. 1. Nuclear morphologies of cells undergoing C-Frag (**A**) and apoptosis (**B**). **A** During C-Frag, mitotic chromosomes are degraded (right part of photo). This is in contrast to normal chromosome morphology (left part of photo). **B** In apoptotic cells, uncondensed chromatin is degraded, forming condensed clusters of nuclear material (top) which are distinct from normal interphase nuclei (bottom).

2011a]. Mitotic catastrophe has been suggested to be a subtype of apoptosis resulting from a failed apoptosis, again suggesting that both forms of cell death are likely pervasive at the same time in response to the same treatment [Castedo et al., 2004a, b; Park et al., 2005, 2007; Galluzzi and Kroemer, 2009]. Thus, overlap of multiple types of cell death is an important issue. Studies based on identification of only one type of cell death are likely to underestimate the amount of cell death taking place, leading to confusion and unreliable treatment expectations.

Heterogeneity of Factors That Induce Death

Cell death can be induced by multiple factors, including changes in local morphogens, hormonal change, signals from neighboring cells, mechanical damage, or drug treatment [Entezari et al., 2010]. Apoptosis occurs as an extrinsic and intrinsic process [Galluzzi et al., 2012]. In extrinsic apoptosis, a number of molecules bind or are removed from receptors. These receptors include FAS, tumor necrosis factor receptor, netrin receptors and others [Galluzzi et al., 2012]. Intrinsic apoptosis is induced by multiple stresses, including many chemotherapeutics and ionizing radiation. Intrinsic apoptosis typically proceeds following mitochondrial outer membrane permeabilization, release of cytochrome c, and activation of apoptosis inducing factor, though it can occur independent of mitochondrial outer membrane permeabilization such as in the case of Bcl-2 or Mcl overexpression [Daugas et al., 2000; Sun et al., 2001; Riedl and Shi, 2004]. Au-

tophagy typically occurs in response to low nutrient abundance as a survival mechanism but can also be induced by various drugs. MCDs also occur in response to various types of stress, often when cell cycle checkpoint function is abrogated, though they can also occur in normal tissue [Stevens et al., 2011a]. C-Frag, for instance, has been shown to occur in swine lymphocytes in response to vaccination [Nichols and Levan, 1965]. It also occurs in response to chemotherapeutic treatments, gene defects, ER stress, HSP90 inhibition, genomic instability and centrosome amplification. Taken together, cell death can be broadly viewed as a response to stress to which cells are unable to adapt [Stevens et al., 2011a].

Heterogeneity of the Cell Death Process

Gene Level

Gene expression in individual cells can be quite heterogeneous [Tantos et al., 2012], and resultant protein levels are also heterogeneous [Sigal et al., 2006]. Expression heterogeneity can be influenced by a multitude of factors, including cell cycle stage, transcriptional activity, differences in signaling in each cell, cell to cell communication, unequal separation of cellular contents during division, mutation, and genomic instability [Cohen et al., 2008]. Among these, cell cycle stage contributes approximately 20% of overall protein expression variability [Cohen et al., 2008]. Expression heterogeneity affects the ability of a cell to use specific pathways. For instance, if apoptotic pathways are inhibited by the lack of caspase expression, alternate cell death pathways and modalities are used. Furthermore, expression of cell death initiating and inhibiting proteins has been shown to occur in response to drug treatment in multimodal patterns; however, even though precise measurements of expression were made over a long time period, most expression changes did not predict outcome [Cohen et al., 2008].

Not only does gene level heterogeneity occur at the expression level, it also occurs at the functional level. Post-translational modifications such as phosphorylation affect the activity and the interaction partners of a given protein, resulting in the propagation of a signal transduction cascade [Baek, 2011]. Other post-translational modifications affect the ability of cells to die. For instance, activating cleavage of some caspases releases inhibitors of apoptosis which in turn can inhibit caspase activity [Darding and Meier, 2012]. Furthermore, the evolutionary conservation of genes results in genes serving more than one function. Caspase 8 may be one such gene. It is

well accepted that caspase 8 functions in the extrinsic apoptosis pathway, but conversely caspase 8 induces proliferation and is required for NF- κ B activation in lymphocytes [Kennedy et al., 1999; Blagosklonny, 2003; Su et al., 2005]. TP53 is another well-known player in forms of cell death that also has many other functions, including transcriptional regulation [Feng et al., 2008; Olivier et al., 2009].

Pathway Level

The gene level heterogeneity discussed above ultimately has its effects on the pathways that function in a cell. For example, the pathways that function in a given cell are not static [Tantos et al., 2012]. These pathways turn on and off, and genes that function in one pathway can have different functions within other pathways. In regards to cell death this means that cells of the same type are capable of utilizing different pathways and undergoing different types of cell death. Furthermore, different networks function in different cell types, often making use of a protein differently than other cell types. Again, different cell types use different pathways and cell death routines to undergo death. The pathway or type of death is largely dependent on the networks that govern the function of a given cell type. Lastly, discussion of pathway level heterogeneity within the context of cell death heterogeneity is prudent. For the convenience of discussion, these types of pathway heterogeneity are listed below. These 3 mechanisms ease the categorization of pathway level heterogeneity, but it should be noted that there can be substantial overlap of these mechanisms. For instance, genome level heterogeneity can contribute to both intrinsic and extrinsic cell death heterogeneity.

Intrinsic Pathway Heterogeneity

Cell death pathway heterogeneity occurs in response to intrinsic factors; for instance, apoptosis can occur in response to intrinsic stimuli such as ROS production, ER stress or genome damage. Heterogeneity plays a role as the mitochondria may or may not be involved, and caspase activity may or may not play a role. When the function of one of these proteins or its related pathways is compromised, other cell death-related pathways can activate, and alternate forms of apoptosis or other cell death types occur.

Extrinsic Pathway Heterogeneity

Extrinsic pathways of apoptosis include signaling pathways that involve Fas and the Fas ligand, the TNF family, and the netrin receptors whose mechanisms are

also heterogeneous. For example, binding of Fas or the TNF family by their ligands results in activation of caspase 8 through recruitment of proteins to the death-inducing signaling complex and the induction of apoptosis [Fuentes-Prior and Salvesen, 2004]. However, removal of netrin induces apoptosis through distinct mechanisms [Mille et al., 2009; Guenebeaud et al., 2010]. Thus, the microenvironment of a cell has an impact on whether or not it dies.

Pathway Heterogeneity Due to Genome Change

Genome level change in cancer is often unique to each cell within a tumor, and genome level alterations are observed even in normal tissues. The population heterogeneity that arises from this genome level change is extensive. The networks that these pathways form are emergent properties, dependent on the specific genome system under which they are organized [Heng, 2009, 2013a, b; Heng et al., 2011a]. In regards to cell death, these changes are expected to impact the cellular system in 2 ways. First, by changing the patterns of genes that are expressed, genome change alters how pathways function. Cell death pathways are not exempt from this. Like other pathways, cell death pathways are far from static in cells with altered genomes, and it is of little surprise that cells within a given heterogeneous population respond in a heterogeneous manner to cell death stimuli. Second, the complexity of apoptotic pathways is astonishing, especially considering that apoptosis is by far the most well characterized form of cell death. It is likely that other forms of cell death show similar complexity.

Genome Level

The functional heterogeneity of genes that play roles in cell death is noteworthy in most normal tissues where healthy cells contain an unchanged genome. This heterogeneity increases in cancer [Heng et al., 2009; Stevens et al., 2011b]. Mutations and genome level alterations such as translocations and aneuploidy occur universally in cancer and impact the heterogeneity of cell death. Mutations occur in a stochastic manner and are often not shared by cells from the same tumor [Bielas and Loeb, 2005]. The observable effects of mutations on cell death are primarily inhibition of cell death, as mutations that induce cell death would most likely not be observable. Genome level change has even greater impact on the heterogeneity of cell death in tumors. The creation of fusion proteins by chromosomal translocations is one such event that has gained notoriety. Fusion proteins have altered function such as the *BCR-ABL* translocation in

chronic myelogenous leukemia which results in a constitutively active ABL kinase that drives proliferation. Other fusion events have been associated with increased proliferation in prostate cancer [Horne et al., 2013a]. Genome level change does not, however, only affect cell death heterogeneity by creating abnormal fusion proteins. Genome level change affects cell death in that it changes expression through changes in copy number and higher level chromatin organization [Heng, 2013a]. One altered chromosome can affect expression levels of tens or hundreds of genes greatly affecting the pathways that a cell can utilize for processes such as death. Thus genome alteration creates a new system reliant on novel pathways. Most tumors are highly heterogeneous at the genome level which in turn promotes cell death heterogeneity within a single tumor [Heng et al., 2010b, 2011b; Stevens et al., 2011a].

Heterogeneity Resulting from Cell Death

The types of cell death that occur, the inputs that lead to it, the genes that contribute to it, and the pathways that control cell death are all heterogeneous, so it should come as no surprise that the results of cell death are also heterogeneous. Typically cell death routinely results in the elimination of a cell; however, cell death processes can be arrested, resulting in an incomplete cell death. Here we will discuss cell death heterogeneity in incomplete and complete cases of cell death.

Incomplete Cell Death

Incomplete cell death is important in a number of physiological processes. For example, caspase 8 activation is required for lymphocyte activation [Su et al., 2005]. Incomplete cell death carried out through activation of caspase 3 is also required for production of granulocytes and megakaryocytes [Galluzzi et al., 2008a]. Other instances of incomplete cell death, however, are not so regulated and create extensive genomic change that can drive cancer progression [Heng et al., 2006a, b, 2011b]. It has been hypothesized that incomplete apoptosis could lead to extensive genome change which may be responsible for a process called genome or karyotypic chaos and recently referred to as chromothripsis where one or more chromosomes have extensive complex translocations (fig. 2) [Heng et al., 2006b; Stephens et al., 2011]. It has been hypothesized that the chromosomal fragments that compose the derivative chromosomes in genome chaos/chromothripsis must stem from chromosomal breaks in mi-

Fig. 2. Incomplete cell death can result in altered genomes. In both panels spectral karyotype images (SKY) are provided on the left, and reverse DAPI images are provided on the right. **A** Incomplete C-Frag can result in the degradation of one or more chromosomes, resulting in aneuploidy. In this figure, the chromosome being degraded is marked by a red arrow in both SKY and DAPI images. Reproduced from Stevens et al. [2007] with permission from *Cancer Research*. **B** Incomplete C-Frag can result in genome chaos when fragments of chromosomes recombine to form highly changed derivative chromosomes (red arrows).

totic cells [Stevens et al., 2007, 2011a; Heng et al., 2011b; Stephens et al., 2011].

Our preliminary data shows that incomplete C-Frag followed by random rejoining of the fragments leads directly to genome chaos (fig. 2) [Liu et al., submitted]. In incomplete C-Frag, chromosomal fragments that are multiple megabases in size are rejoined apparently randomly. This leads to the production of amalgamated chromosomes composed of multiple fragments from 2 or more parental chromosomes. Incomplete C-Frag and autophagy can also degrade a single chromosome which in turn leads to aneuploidy [Sit et al., 1996; Stevens et al., 2007]. Thus, induction of cell death can in some cases lead to the alteration of a targeted cell, increasing the evolutionary potential of that cell and the probability that eventually a resistant cell will come about.

Completed Cell Death

Problems resulting from inappropriate cell death also arise from completed cell death. Most molecular research focuses on the process of cell death, but how the cell dies is not the only important part of the cell death story. While

the cell death process is a response to stress, it can also cause new stress to the system. For example, the release of cellular contents upon necrotic cell death elicits an immune response which can damage and destroy neighboring cells. This immune reaction involves release of various ROS species into the extracellular space which can induce mutations and genome change, which ultimately introduces heterogeneity into the surrounding cells. In contrast to necrosis, during apoptosis cellular contents are packaged into blebs surrounded by cellular membrane to facilitate uptake by neighboring cells and avoid an immune response. While apoptosis does negate an immune response, it has also been shown to facilitate horizontal transfer of DNA that is packaged into the blebs [Bergsmedh et al., 2001]. This can result in genome change or copy number changes which are especially deleterious if these might be powerful oncogenes. Furthermore, for apoptotic cells to escape immune activation, apoptotic cells must be engulfed by neighboring cells or macrophages where they can be digested. Disruption to the engulfment of apoptotic cells has been implicated in autoimmune diseases such as lupus and arthritis [Nagata et al., 2010].

Fig. 3. Proposed model of the relationship between heterogeneity and various types of cell death and its potential impact on genome variation. Cell death heterogeneity as viewed from 3 key levels including heterogeneity of induction of cell death, processes and outcomes. Cell death heterogeneity is linked with different types of cell death and also serves as a source of genome alteration that is necessary material for somatic macro-evolution.

Implications of Cell Death Heterogeneity

Much of the current work in the cell death field is based on reductionist concepts and approaches. These concepts have been central to the development of our current understanding of cell death. The field has successfully linked many genes to various cell death pathways. It is true that each of these pathways can lead to cell death; however, the clinical utility of the continued identification of genes associated with cell death is limited when multiple types of cell death occur and a large number of pathways exist for each type of cell death. Thus, if nearly every pathway within a cell can be linked to cell death, what is the value of one isolated pathway, and how can switches in the pathways that are activated for cell death be predicted? In order to truly make an advance in cell death research, we must augment the currently known pathways and responses with the true complexity of living systems. To do so, multiple mathematical models are often applied to a biological function such as cell death. Multiple modeling techniques have been applied to increase understanding of apoptosis [Spencer and Sorger, 2011]. These models include ordinary differential equations, stochastic models, Boolean models, stability analysis, among others [Chen et al., 2007, 2010; Luan et al., 2007; Calzone et al., 2010]. An emerging concept to deal with this complexity is to study the evolutionary mechanism of a biological process rather than cataloging each minute molecular detail involved in that process. Studies of the overall impact of stress from a more systematic point of view may allow us to identify differences in system behavior(s) that better reflect changes in the overall

state of the system. For example, we have shown that the evolutionary mechanism of cancer can be described as the sum of all molecular mechanisms and that the evolutionary potential of cancer is measured by genomic instability at the chromosome level [Heng, 2009; Heng et al., 2010b, 2011b]. It was subsequently shown that focus on total genome change rather than specific gene alterations is a better predictor of cancer progression. Similarly we have shown that the evolutionary mechanism of C-Frag is simply a cellular response to stress (fig. 3).

Basic Research

Because of the overwhelming heterogeneity of cell death in populations, simply monitoring the expression or activation of a protein in an extraction from a mixed population of cells is not sufficient to explain what is happening to the entire population. Many of the basic tools of molecular biology rely on profiles created from a population average. For instance, Western blots show the relative expression of a protein in a mixture of proteins derived from the lysis of a population of cells. According to our recent data, the average growth of a population is not driven by average cells from the population, but rather by highly proliferative outliers, especially in cases of high genome heterogeneity [Heng et al., 2010a, b; Abdallah et al., unpublished data]. Methods that average a given parameter, such as Western blotting, also may hide information about the processes that occur. These measurements have been used to show that induction of caspase activation following treatment by inducers of apoptosis takes place gradually. In contrast, apoptotic events occur rapidly following a prolonged period of stasis following the initial

treatment [Goldstein et al., 2000a, b; Tyas et al., 2000]. Thus, profiling the population average is often deceiving. Furthermore, biochemical studies must be undertaken with caution as some aspects of cell death held to be almost akin to biological laws do not always hold true. For instance, apoptosis can occur without caspase involvement, and exposure of phosphatidylserine on the outer cell membrane can indicate that other forms of death have occurred [Mihalache et al., 2011]. Similarly, reliance on only morphological criteria can miss the underlying biochemical processes occurring during that death [Galluzzi et al., 2012]. In a given tumor or population, individual knowledge of the specific processes may enable a more robust induction of cell death.

Cellular heterogeneity impacts longer term results. Frequently studies of cell death focus on a short time window of either a few hours or a few days. However, studies of the results of a drug in a short time window may not be representative of the effects of heterogeneity over time. For example, if a targeted agent is applied to a heterogeneous population of cells, cells expressing the target will be eliminated over a given time period. Molecular analysis may also indicate a strong death response, but cells within the population that lack the target would be spared. Over time these few survivors repopulate the tumor, leading to resistance. In practice this is much more complex, as a drug treatment can induce adaptive genome change within the population. Thus, it is prudent to monitor the response of cells for longer periods of time, weeks, if not months.

An important aspect of cell death is the portion of the cell cycle in which the death occurs. The portion of the cell cycle in which the death occurs is often ignored but may give insight into the status of a cell. For example, in normal, healthy cells, cell cycle checkpoints exist to ensure that the cell is unstressed and important events such as DNA replication have completed. Prolonged activation of these checkpoints can lead to activation of cell death pathways. Checkpoint integrity in cancer is typically compromised, and cells often enter mitosis with extensive damage. When this occurs, a cell with sufficient damage will undergo MCD directly in mitosis via C-Frag or following an abortive mitosis via mitotic catastrophe.

In the Clinic

The behavior of individual cells within a population in response to treatment is very important. Measurements of reduction in tumor volume often do not indicate long term outcome. Initial reduction of a tumor burden may be a desirable goal, but treatment must be balanced so

that the cell death that is induced is largely tumor-specific and has little off-target effects [Heng et al., 2010a; Heng, 2013b]. Furthermore, care must be taken in treatment design to avoid induction of genomic instability and subsequent increases in the evolutionary potential of a tumor. Treatments must be designed to favor the elimination of abnormal cells in diseases such as cancer to slow the disease process, while avoiding treatment induced system instability such as genome chaos.

Conclusion

Cell death heterogeneity is an important issue and is responsible for much confusion in cell death research. This is especially true in regards to the effectiveness of various cancer therapies at inducing cell death. Cell death heterogeneity often causes inconsistent outcomes, even when the same treatment is applied to the same system. While the cell death research field has had great success in identifying molecules involved in cell death, cell death is still far from being understood. Developing a deeper understanding of the multiple levels of heterogeneity in cell death is central to pushing a new era of cell death research. To accomplish this, new approaches and ideas are required that embrace complexity and eschew reliance on linear models.

Many biological questions cannot be answered with a simple yes or no, and cell death is no exception to this. Cell death is not good or bad, but rather can be both. However, in the cancer field, focus is often placed on one pathway or event in a population that is thought to be a positive marker, such as the appearance of caspase activity, which is deemed as a good result of treatment. If cells die appropriately, cell death usually occurs in a beneficial manner, and this cell death is good. If it occurs inappropriately or is incomplete, it is detrimental. Along with changes to the underlying framework of cell death research, cell death heterogeneity requires the development of new experimental and statistical tools [Heng et al., 2010a, b]. Complex statistical methods, such as multimodal analyses, are required to understand the effects of outliers. Studies must also be adapted to include longer time windows in order to appreciate phenomena such as how incomplete cell death can promote genomic diversity and long term survival while in the short term reduce tumor volume [Heng et al., 2010a]. Cell cycle information is also an important addition to future research as it can aid in the determination of the types of cell death that are occurring [Stevens et al., 2007, 2011a]. Importantly, we

have to consider what is being measured. For instance, if caspase cascades are strongly activated but a treated tumor persists after a few weeks, are the correct metrics being assessed? We have shown that broadly defined stress is linked to cell death in general, so the focus should be on the ultimate phenotype of death, rather than on specific molecules whose interactions will diminish our ability to harness cell death in a constructive manner. We have previously introduced a similar idea to cancer research in which we use random rather than specific genome change to monitor genomic instability [Heng et al., 2006a, b]. This method has proven to be a more robust measure of tumorigenic potential than accounting for specific gene mutations or pathways. Finally, genome alteration is linked to multiple diseases [Iourov et al., 2008; Heng,

2010; Sgaramella, 2010] and plays an important role in organismal evolution [Heng et al., 2006b; Gorelick and Heng, 2011; Heng, 2013b; Horne et al., 2013b]. Therefore, cell death heterogeneity clearly requires more attention in regards to somatic and organismal evolution.

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